Electromechanical stimulation ameliorates inactivity-induced adaptations in the medial gastrocnemius of adult rats


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The purpose of the present study was to compare the impact of a neuromuscular stimulation paradigm delivered in one vs. two sessions per day on the size, functional, and phenotypic properties of the rat medial gastrocnemius (MG; a plantar flexor muscle composed predominantly of fast fibers) in SI animals. Given that we are attempting to identify the least amount of stimulation needed to sustain normal muscle mass and physiological properties, we chose to study a relatively fast extensor muscle, i.e., the MG, because predominantly fast muscles presumably need relatively small amounts of activation to sustain their properties compared with slow muscles and more atrophy occurs compared with relatively fast flexor muscles in response to decreased activity or inactivity (17, 32). It seems highly likely that the “dose-response” curve will differ among muscle types. Based on the normal activation level and/or pattern of the MG during routine movements (36) and during cage activity (22, 28), we hypothesized that brief bouts of high-load, short-duration stimulation during the initial 30 days after SI would be sufficient to lessen the inactivity-induced adaptations in its mass and functional capacity. Furthermore, we hypothesized that this stimulation paradigm de-

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livered in two sessions per day would result in a more positive net protein status within a given 24-h period and less muscle atrophy and phenotype changes associated with the loss of neuromuscular activity compared with the same amount of stimulation delivered in one session per day. The results, in general, are consistent with these hypotheses.

MATERIALS AND METHODS

Experimental groups. Two identical studies were performed to provide tissues for the physiological and cellular analyses. Adult female Sprague-Dawley rats (223 ± 3 g body wt for the physiological study and 229 ± 2 g body wt for the cellular study) were assigned randomly to a normal control (Con), SI, SI-Sham, SI-P1, or SI-P2 group (n = 6 for each group in both studies). All SI rats underwent SI surgery. In addition to the SI surgery, rats were implanted unilaterally with a nonfunctional (SI-Sham) or functional (SI-P1 and SI-P2) microstimulator, respectively. Rats in the SI-P1 (protocol 1) and SI-P2 (protocol 2) groups received unilateral electromechanical hindlimb stimulation training (SI-Stim1 and SI-Stim2, respectively), while the contralateral limb served as an internal control (SI-C1 and SI-C2, respectively).

Surgical procedures and animal care. The SI surgery procedure was a modification (18) of the original protocols of Tower (47). Rats undergoing SI surgery (SI, SI-Sham, SI-P1, and SI-P2 groups) were anesthetized with intraperitoneal injections of a combination of ketamine hydrochloride (100 mg/kg body wt) and xylazine (5 mg/kg body wt). The spinal cord was transected completely at both a midthoracic and a high-sacral spinal cord level, and a subdural bilateral dorsal rhizotomy was performed between the two transection sites.

During the same surgery, rats were implanted unilaterally with a BION microstimulator capsule missing its electrical components (SI-Sham group) or a functional BION microstimulator (SI-P1 and SI-P2 groups). The BION microstimulator was inserted parallel to the sciatic nerve (along the lateral thigh of the hindlimb between the biceps femoris and the vastus lateralis) with the anode positioned rostrally and the cathode positioned caudally. The proper placement of the BION was verified for each rat in the terminal experiment. All surgical procedures were performed under aseptic conditions.

PolyFlex, a general antibiotic, and Buprenex, an analgesic, were administered (150 mg/kg and 0.05 mg/kg sc twice daily, respectively) during the first 3 days of recovery. Postsurgical care involved manual expression of the bladder. If a rat developed a urinary tract infection, it was administered Baytril (40 mg/kg) in its water for 5 days. The hindlimbs were manipulated passively once a day through a full range of motion to prevent joint fixation, and reflexes in the hindlimbs (i.e., withdrawal reflex and toe spread response) were assessed daily. Throughout the study, there was no response to reflex testing or toe pinching, and the hindlimbs remained completely flaccid. Rats in the Con group were maintained under the same housing conditions. The care and maintenance of SI animals has been detailed previously (35). All procedures were approved by the UCLA Chancellor’s Animal Research Committee and followed the American Physiological Society Animal Care Guidelines.

Electromechanical stimulation. The BION microstimulator is a wireless implantable (2-mm diameter × 16-mm length) radio frequency-powered device designed for functional electrical stimulation. It is composed of a hermetically sealed ceramic capsule containing an electronic subassembly (29). Once implanted, the BION microstimulator receives power and stimulation commands (pulse width, pulse amplitude, frequency, stimulation duration, and cycle time) via a 2-MHz magnetic field generated by an external radio frequency coil that is connected to a BION Control Unit. To ensure proper function, metallic material was kept at least 10 cm away from the coil during use.

During electromechanical stimulation training of the SI-P1 and SI-P2 rats, the limb implanted with a BION microstimulator was secured in a removable cast (ankle at ~90° and knee at ~120°) to optimize isometric contractions of the musculature functioning at the ankle and knee, including the MG. A force transducer embedded in the plantar surface of the cast enabled quantification of the torque at the ankle produced by each stimulation and was used to monitor the tension production throughout each stimulation bout. One bout of stimulation consisted of a 1-s maximum tetanic isometric contraction (100 Hz) delivered once every 30 s for 5 min, followed by 5 min of rest (during which the limb was removed from the cast) (Fig. 1A). The SI-Stim1 limb from the SI-P1 group received a single session of six consecutive bouts of stimulation (protocol 1; Fig. 1B). The SI-Stim2 limb from the SI-P2 group received two sessions of three consecutive bouts of stimulation with the sessions separated by a 9-h interval (protocol 2; Fig. 1C). The stimulation pulse width was maintained at 50 μs, and the threshold pulse amplitude (μA) was determined daily by doubling the minimum pulse amplitude necessary to produce a maximum twitch response. Pulse amplitude was increased as necessary to maintain maximum tetanic tension throughout a session. The torque generated across the ankle with each stimulation pulse was monitored. At least 90% of the tension produced at the start of each stimulation bout was preserved at the end of the bout with the above protocols.

Daily stimulation training for both SI-P1 and SI-P2 groups began 2 days after surgery and continued for 30 consecutive days. For both groups the total duration of the training sessions was 1 h/day, while the total stimulation duration was 1 min or 0.069% of a 24-h period.

In situ mechanical properties. The MG muscles from Con, SI, SI-Sham, and SI-P1 and SI-P2 groups in the physiological study were tested for their in situ mechanical properties as described by Roy et al. (39). The MG tendon was secured to a stiff wire near the musculotendinous junction and attached to a force-transducer (Satham, Gould, Cleveland, OH) or a force transducer-lever system (Cambridge Instruments, Taunton, MA) to measure the isotropic and isotonic properties of the muscle, respectively.

The average time-to-peak-tension (TPT), half relaxation time (HRT), and maximum twitch tension (Pt) were determined from 10 consecutive isometric twitch responses at the optimum muscle length (L0). Peak isometric tension development was measured at frequencies of 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 200, and 300 Hz for a duration (between 300 and 500 ms) that resulted in a plateau in the frequency-tension curve. The highest tension achieved, regardless of
The maximum shortening velocity ($V_{\text{max}}$) was obtained at $L_o$ for 15–20 afterloaded contractions (100-ms tetanus at 200 Hz) at various loads of <30% of $P_o$.

For each isotonic contraction, peak velocity and the associated force were plotted as $y = (P_o - P)/V$ vs. $x = P$, where $V$ is the measured velocity and $P$ is the measured force. A regression line was fit to the data, and Hill’s (21) constants $a$ and $b$ were determined. The absolute maximum rate of shortening ($V_{\text{max}}$, mm/s) was calculated as $P_o$ multiplied by the inverse of the slope and divided by the $\gamma$-intercept extrapolated by the regression analysis. In addition, $V_{\text{max}}$ was expressed relative to the muscle length (ML) measured at $L_o$ at the end of the in situ testing (MLs). Force-velocity curves were generated using Hill’s coefficients $a$ and $b$ as previously described (44). Power-velocity curves then were generated from these force and velocity data to determine the maximal power output.

The mean in situ MG muscle lengths at $L_o$ (33.6, 28.6, 29.9, 28.9, and 32.1 mm for Con, SI-C1, SI-C2, SI-Stim1, and SI-Stim2 groups, respectively) were used to estimate fiber lengths based on reported muscle length-to-fiber length ratios (33). The estimated fiber lengths (12.4, 10.6, 11.0, 10.7, and 11.9 mm, respectively) were used to calculate the physiological cross-sectional area (PCSA) using the following formula:

$$\text{PCSA} = \frac{(\text{mass~muscle}) \times \text{cosine theta}}{(\text{fiber~length}) \times \text{muscle~density}}$$

where the muscle mass was the muscle wet weight (g), theta was equal to the approximate angle of fiber pennation in one dimension (33), and a muscle density of 1.056 g/cm$^3$ was assumed (34). Specific tension (SpT; kg/cm$^2$) was calculated as maximum isometric tetanic tension produced during mechanical property measurements divided by PCSA (39).

**Tissue harvesting.** A number of hindlimb muscles, including the MG, were removed bilaterally, trimmed of fat and connective tissue and weighed (wet weight). The MG muscles from the physiological testing were harvested from the midbelly of the MG, and stored at −80°C until used for immunohistochemical analyses. MG muscles from the cell

**Immunohistochemical analyses.** Serial cross sections (10 μm thick) from the midbelly of the MG were used to determine the MHC profiles of −100 adjacent fibers from a representative area of the deep (MGd, the region of the muscle near the tibia that contained some slow fibers) and superficial (MGs, the region of the muscle away from the tibia that was comprised exclusively of fast fibers) regions. A series of monoclonal antibodies specific to rat MHC isoforms were used for the immunohistochemical analyses as described by Talmadge et al. (43). The anti-slow, anti-fast, and anti-developmental antibodies (Vector Laboratories, Burlingame, CA) were used to identify the slow, fast, and developmental (embryonic and neonatal) MHC isoforms, respectively. The F3 antibody, kindly provided by Dr. Schiaffino (Padova, Italy) (40), is specific for the IIb MHC isoform. The avidin-biotin immunohistochemical procedure was used to localize and amplify the antigen-antibody binding complex ( Vectastain ABC kits, Vector Laboratories). Additional tissue sections were stained without primary antibody incubation to control for nonspecific binding. Fibers were considered to contain a specific MHC if there was a visually detectable reaction in the fiber to the appropriate monoclonal antibody. Fibers were classified as type I (positive reaction exclusively for the slow antibody), type I+ IIa/x (positive for both the slow and fast, and negative for the F3, antibodies), type IIa/x (positive exclusively for the fast antibody), and type IIb+ (positive for both the fast and F3 antibodies and thus expressing IIb and possibly Ila and/or IIX MHC). All fibers were negative for the developmental antibody. The cross-sectional areas (CSAs) of all type-identified fibers were determined using the Scion image processing system (FREDERICK, MD) from laminin (Vector Laboratories)-stained sections. The number of fibers measured to determine the mean CSA of each fiber type was dependent on the percentage of that fiber type identified in each region of the MG.

The relative contribution of the deep and superficial regions of the MG to the total CSA was determined using a planimeter. Weighted mean CSA was calculated for each region of each muscle by summing the products of the mean fiber CSA for each fiber type by the percentage of that fiber type present within the area. The product of the mean fiber CSA and the percentage of the given fiber type then was divided by the weighted mean CSA. The value for each fiber type in each region was calculated and summed to determine the relative contribution of the fiber type to the whole muscle CSA.

**Biochemical and molecular analyses.** As previously described by Haddad et al. (20), a portion from the midbelly of the MG (mixed region) was cut, weighed, and homogenized in 20 volumes of homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 10 mM Tris base). A known volume of the total homogenate was used for myofibrillar protein extraction using a modification of the original protocols of Solaro et al. (41). The extracted myofibrils were suspended in a known volume of 100 mM KCl, 10 mM Tris, and 1 mM EDTA, pH 7.4. The protein concentration of the total homogenate and myofibrillar suspension were determined by the Bio-Rad protein assay and were used to calculate the total protein and myofibrillar protein concentrations of the MG. The total homogenate then was diluted to a final protein concentration of 1 mg/ml in a storage buffer (50% glycerol, 100 mM Na$_2$PO$_4$, 5 mM EDTA, and 2 mM 2-mercaptoethanol, pH 8.8) and stored at −20°C. DNA concentration was measured using a volume of the total homogenate and a fluorometric assay (27) and then used to calculate the total DNA concentration of the MG. The concentration values were used to calculate total protein, myofibril, and DNA content by multiplying by MG weight.

**MHC and actin protein.** The MHC and actin proteins from the total protein homogenate were separated on an acrylamide gel using the SDS-PAGE technique. Ten-microgram volumes of protein sample were denatured in 40 μl of sample buffer (5% β-mercaptoethanol, 100 mM Tris base, 5% glycerol, 4% SDS, and 0.05% bromophenol blue, pH 6.8) by heating at 100°C for 2 min. Fifteen microliters of the denatured protein were loaded per lane (equivalent to 3 μg of total muscle protein) along with purified myosin and α-skeletal actin (the ~42-kDa band that comigrates with purified α-skeletal actin) (Sigma Chemical, St. Louis, MO) as standards. Following electrophoresis, the gels were stained with brilliant blue (Sigma Chemical), destained, and scanned with a Molecular Dynamics (Sunnyvale, CA) densitometer. The MHC and actin band intensities were quantified using the Image Quant Software. The MHC and actin proteins were expressed as arbitrary units (AU) per microgram of total protein, and the amount of MHC and actin protein content was calculated based on muscle total protein content (20).

**SDS-PAGE.** The total protein homogenate was boiled in sample buffer for 2 min and loaded on an SDS-PAGE gel where the MHC isoforms were separated according to Talmadge and Roy (42). The gels were run at 275 V for ~20 h under refrigeration then stained with brilliant blue (Sigma Chemical), destained, and scanned with a Molecular Dynamics (Sunnyvale, CA) densitometer. The area of each MHC isoform band was identified, and the integration was determined using the Image Quant Software.

**Statistical analyses.** All data are presented as means ± SE. The normality of each outcome variable was determined and a logarithm transformation (or square root transformation if there were zero values) was applied before group comparisons if the distribution was skewed. Because the outcomes were obtained from both the stimulated and nonstimulated limbs of each SI rat, a mixed model was used to take the intraindividual correlation into account and to determine any overall group differences, where the group was the fixed effect and the rat was the random effect in the model. This was followed by pairwise comparisons when there was an overall group effect. Percent change data of the stimulated from the nonstimulated limb were
compared between the two protocols using the Wilcoxon rank sum test. The level of significance was set at $P \leq 0.05$.

**RESULTS**

The effects of inactivity on the SI, SI-Sham (both implanted and nonimplanted limbs), and the contralateral nonstimulated MG from the SI-P1 and SI-P2 groups were similar for all measurements, indicating that the implant surgery of the BION microstimulator had no significant effects on the measured muscle properties. Therefore, any differences observed between the stimulated and nonstimulated MG of SI rats are due to the electromechanical stimulation training. Furthermore, data from the contralateral nonstimulated MG of the SI-P1 and SI-P2 groups (SI-C1 and SI-C2, respectively) serve as effective internal controls and will be used to represent the effects of inactivity hereafter.

**Body and muscle weights.** Initial mean body weights from the physiological study were similar across all groups (Con, SI-P1, and SI-P2 groups were $215 \pm 3$, $227 \pm 4$, and $224 \pm 5$ g, respectively). At the end of the 30-day study, the Con, SI-P1, and SI-P2 groups had gained 37%, lost 9%, and had no change in body weight, respectively. The mean terminal body weights of both SI-P1 and SI-P2 groups were lower than the Con group, and higher in the SI-P2 than SI-P1 group. The absolute MG weights were 45 and 49% of Con in the SI-C1 and SI-C2 groups, respectively (Fig. 2A), and the relative weights (to body weight) were $\sim 63\%$ of Con in both SI-C groups (Fig. 2B). In both SI-Stim groups, the MG was larger in the stimulated than the nonstimulated limb. The absolute (Fig. 2A) and relative (Fig. 2B) MG weights were 13% larger in the SI-Stim1 group, and 26% larger in the SI-Stim2 group, respectively, compared with their SI controls. Furthermore, the absolute and relative MG weights were 23 and 10% larger in the SI-Stim2 than the SI-Stim1 group, respectively.

**Physiological properties.** Mean $P_o$ was lower than Con in all SI groups (Fig. 3A). Mean $P_o$ was 40 and 44% of Con in the SI-C1 and SI-C2 groups, and 60 and 73% of Con in the SI-Stim1 and SI-Stim2 groups, respectively. The mean $P_o$ was higher (21%) in the SI-Stim2 than SI-Stim1 group. Overall muscle function (mean $P_o$ relative to body mass) was significantly improved with stimulation, with SI-C, SI-Stim1, and SI-Stim2 group values that were 56, 85, and 93% of Con, respectively. Mean $P_t$ was lower than Con in all SI groups, and it was similar for the SI-Stim and their respective SI-C groups (Table 1). Unexpectedly, $P_t$ was lower (27%) in the SI-Stim2 than SI-Stim1 group, suggesting that the two stimulation sessions per day paradigm depressed the active state of the muscle. Mean $P_t/P_o$ ratios were higher in the SI-C1 and SI-C2 than the Con group, whereas this ratio was similar to Con in the two SI-Stim groups. In addition, the $P_t/P_o$ ratios were lower in the SI-Stim2 than SI-Stim1 group. The $P_t/P_o$ ratios reflect the larger impact of both SI and stimulation on tetanic vs. twitch tension capability. Mean twitch TPT and HRT for each SI-Stim and its respective SI-C group were similar (Table 1). Thus it appears that neither stimulation paradigm had any effect on

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**Fig. 2.** Absolute (A) and relative (to body weight; B) medial gastrocnemius (MG) weights from control (Con); spinal cord-isolated nonstimulated control for protocol 1 (SI-C1), spinal cord-isolated stimulated group for protocol 1 (SI-Stim1), spinal cord-isolated nonstimulated control for protocol 2 (SI-C2), and spinal cord-isolated stimulated group for protocol 2 (SI-Stim2) groups in the physiological study. Values are means ± SE. Significant difference from Con, $P \leq 0.05$. †Significant difference from contralateral SI-C, $P \leq 0.05$. ‡Significant difference from SI-Stim1, $P \leq 0.05$.

**Fig. 3.** Maximum tetanic tension (A) and specific tension (to body weight; B) of the MG from Con, SI-C1, SI-Stim1, SI-C2, and SI-Stim2 groups at the end of the 30-day physiological study. Specific tension is expressed as tension (kg) produced per physiological cross-sectional area (cm$^2$). Values are means ± SE. *Significant difference from Con, $P \leq 0.05$. †Significant difference from contralateral SI-C, $P \leq 0.05$. ‡Significant difference from SI-Stim1, $P \leq 0.05$.
sarcoplasmic reticulum calcium kinetics during a single stimulus.

Mean PCSA was smaller in all SI groups than in the Con group (Table 1). Mean PCSA was 12 and 18% larger in the SI-Stim1 and SI-Stim2 groups compared with their contralateral controls, respectively. The mean SpT was ~23% lower in the SI-C1 and SI-C2 groups than the Con group (Fig. 3B). Mean SpT in both Stim groups, however, was similar to Con, and it was higher than in their respective contralateral nonstimulated muscles. Similarly, mean peak power was lower in the nonstimulated groups than in the Con and SI-Stim groups (Table 1).

The absolute amount of force produced at all frequencies of stimulation tested was lower in all SI groups compared with Con (Fig. 4A). Both stimulation paradigms resulted in forces closer to control values than their contralateral counterparts at most frequencies of stimulation, with a greater efficacy with the SI-Stim2 compared with the SI-Stim1 paradigm. The absolute tension produced at all frequencies >30 Hz was larger in the SI-Stim1 than the SI-C1 group. The absolute tension produced at 25 Hz and all frequencies >40 Hz was larger in the SI-Stim2 than the SI-C2 group (Fig. 4, A and B). Furthermore, the tension produced at 5 Hz and all frequencies above 75 Hz was higher in the SI-Stim2 than the SI-Stim1 group.

When expressed relative to Po, the tensions were higher (curve shifted to the left) in the SI-C than the Con group at the lower frequencies (up to 25 and 40 Hz in the SI-C1 and SI-C2 groups, respectively; Fig. 4C). In contrast, the SI-Stim1 curve was similar to Con, and the SI-Stim2 curve was shifted to the right of both the Con (at 40 and 50 Hz) and the SI-Stim1 (at all frequencies up to 50 Hz, except 30 Hz) curves. The SI-Stim1 (at all frequencies up to 25 Hz) and SI-Stim2 (at all frequencies up to 75 Hz) curves were shifted to the right of the curves for their contralateral nonstimulated muscles. Thus the MG muscles of SI rats produced a higher percentage of their maximum force at lower stimulation frequencies and this effect was reversed with stimulation. The leftward shifts in the curve (indicating a shift toward “slower” properties) for the SI groups were unexpected. Mean V_max (ML/s) values in SI-C1, SI-C2, and SI-Stim1 groups were similar to Con values and lower than Con and SI-Stim1 in the SI-Stim2 group (Table 1).

Cellular properties. The initial mean body weights from the cellular study were similar across Con, SI-P1, and SI-P2 groups (224 ± 3, 233 ± 5, and 230 ± 4 g, respectively). At the end of the 30-day study, Con, SI-P1, and SI-P2 groups gained 25%, had no change, and lost 16% body weight, respectively. This resulted in lower mean terminal body weights for both SI-P1 and SI-P2 groups compared with the Con group, and a higher mean terminal body weight in the SI-P1 than the SI-P2 group. The absolute MG weights in Con, SI-C1, and SI-C2 groups were 684 ± 14, 404 ± 30, and 344 ± 13 mg, respectively. Thus absolute MG weights were 59 and 50% of Con and relative MG weights were 76 and 73% of Con in the SI-C1 and SI-C2 groups, respectively. The absolute and relative MG weights of the SI-Stim1 group were 11% larger than in the SI-C1 group and were 21% larger in the SI-Stim2 group (Fig. 5A).

The general effects of SI and electromechanical stimulation training on muscle mass were similar for the physiological and cellular studies. However, unlike the other SI groups, the SI-P2 group from the cellular study failed to gain weight over the course of the 30-day following the initial rapid loss in body weight observed during the first 10 to 14 days of SI. This resulted in lower absolute muscle weights for this group. Nonetheless, the greater efficacy of the SI-Stim2 protocol compared with the SI-Stim1 protocol as a countermeasure for muscle atrophy was still evident when comparing the stimulated limbs with their respective internal controls. For example, the relative MG weight of the SI-Stim1 group was 13 and 11% larger than their contralateral controls in the physiological and cellular studies, respectively. The SI-Stim2 group was 26 and 21% larger than the SI-C2 group in the physiological and cellular studies, respectively. Consequently, the most appropriate measure of the effects of electromechanical stimulation training and comparisons of efficacy between the two training protocols is a comparison of the stimulated limbs (SI-Stim1 and SI-Stim2) with their internal SI controls (SI-C1 and SI-C2). Therefore, although we present the mean values in the following text, the effects of stimulation on the cellular properties of the MG are presented as the percent change from their respective SI controls in the accompanying figures.

The total protein concentration in the MG was similar to Con (232 ± 4 mg/g) in the SI-C1, SI-Stim1, and SI-Stim2 groups (224 ± 5, 231 ± 8, and, 223 ± 6 mg/g, respectively), and it was lower than Con in the SI-C2 group (196 ± 6 mg/g). This resulted in a 14% increase in total protein concentration in the SI-Stim2 compared with the SI-C2 group, and no change between the SI-Stim1 and SI-C1 group (Fig. 5A). The total protein content was lower in all SI groups (90 ± 6, 68 ± 4, 103 ± 7, and 93 ± 4 mg/muscle in SI-C1, SI-C2, SI-Stim1, and SI-Stim2 groups, respectively) compared with Con (159 ± 4 mg/muscle). Stimulation training increased protein content

### Table 1. Mechanical properties of the rat MG muscle from the physiological study

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>SI-C1</th>
<th>SI-Stim1</th>
<th>SI-C2</th>
<th>SI-Stim2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological cross-sectional area, cm^2</td>
<td>0.625±0.019</td>
<td>0.330±0.011*‡</td>
<td>0.370±0.021*‡</td>
<td>0.346±0.015*</td>
<td>0.408±0.011*‡</td>
</tr>
<tr>
<td>Maximum twitch tension, g</td>
<td>233±12</td>
<td>146±8*</td>
<td>174±14†</td>
<td>152±14*</td>
<td>127±9†‡</td>
</tr>
<tr>
<td>Twitch-to-tetanic ratio</td>
<td>0.14±0.01</td>
<td>0.23±0.03*</td>
<td>0.17±0.01†</td>
<td>0.22±0.02*</td>
<td>0.11±0.01‡</td>
</tr>
<tr>
<td>Time to peak tension, ms</td>
<td>21.5±1.3</td>
<td>23.3±1.0</td>
<td>25.2±0.4*</td>
<td>18.4±0.8*</td>
<td>18.2±3*‡</td>
</tr>
<tr>
<td>Half relaxation time, ms</td>
<td>11.7±0.6</td>
<td>13.0±0.8</td>
<td>14.0±0.3*</td>
<td>10.2±0.7</td>
<td>10.3±0.5‡</td>
</tr>
<tr>
<td>Maximum rate of shortening, ML/s</td>
<td>7.1±0.3</td>
<td>7.1±0.5</td>
<td>7.0±0.3</td>
<td>6.9±0.3</td>
<td>6.0±0.2‡</td>
</tr>
<tr>
<td>Peak power, W/kg</td>
<td>528±19</td>
<td>353±17*</td>
<td>465±17*‡</td>
<td>431±28*</td>
<td>479±20*‡</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 rats/group. Con, control; SI-C1 spinal cord-isolated nonstimulated control for protocol 1; SI-Stim1, spinal cord-isolated stimulated group for protocol 1; SI-C2, spinal cord-isolated nonstimulated control for protocol 2; SI-Stim2, spinal cord-isolated stimulated group for protocol 2; MG, medial gastrocnemius; ML, muscle length. *Significant difference from Con, P ≤ 0.05. †Significant difference from contralateral SI-C, P ≤ 0.05. ‡Significant difference from SI-Stim1, P ≤ 0.05.
by 15% and 40% in the SI-Stim1 and SI-Stim2 groups compared with their contralateral controls, respectively (Fig. 5A). The myofibril protein content was lower in all SI groups (35 ± 2, 25 ± 2, 39 ± 4, and 37 ± 3 mg/muscle in SI-C1, SI-C2, SI-Stim1, and SI-Stim2 groups, respectively) compared with Con (66 ± 5 mg/muscle). Compared with their respective contralateral controls, stimulation training increased myofibril protein content by 50% in the SI-Stim2 group and had no effect on the SI-Stim1 group (Fig. 5A). Therefore, the effect of stimulation on total myofibril content and concentration was greater in the SI-Stim2 compared with the SI-Stim1 group.

Fig. 5. Mass and cellular properties of the MG at the end of the 30-day cellular study. A: relative MG mass (mg/g), protein concentration (mg/g) and content (mg/muscle), and myofibril concentration (mg/g) and content (mg/muscle). B: total myosin heavy chain [MHC; arbitrary units (AU)/µg protein], MHC protein content (AU/muscle), α-skeletal actin (α-Sk actin; AU/µg protein), and actin protein content (AU/muscle). C: DNA concentration (mg/g) and content (mg/muscle), and protein-to-DNA ratio (mg/µg). Data are presented as percent change of SI-Stim1 and SI-Stim2 groups from their contralateral SI controls, i.e., SI-C1 and SI-C2, respectively. Values are means ± SE. †Significant difference from contralateral SI-C, P < 0.05. ‡Significant difference from SI-Stim1, P < 0.05.

Fig. 4. Absolute (A) and relative [% of maximum tetanic tension (P_max); C] frequency of stimulation-tension responses for the MG muscle from Con, SI-C1, SI-Stim1, SI-C2, and SI-Stim2 groups at the end of the 30-day physiological study. B: expanded absolute frequency of stimulation-tension response from 5 to 30 Hz from A. Values are means ± SE. *Significant difference of all SI groups from Con, P ≤ 0.05. †Significant differences of SI-C1, SI-C2, and SI-Stim2 groups from Con, respectively, P ≤ 0.05. ‡Significant differences of SI-Stim1 and SI-Stim2 from their contralateral SI-C groups, respectively, P ≤ 0.05. §Significant difference of SI-Stim2 from SI-Stim1, P ≤ 0.05.
The total MHC protein fraction was lower than Con (27 ± 1 AU/μg protein) in the SI-C1, SI-C2, and SI-Stim1 (~23 AU/μg protein) groups, and it was similar to Con in the SI-Stim2 (26 ± 1 AU/μg protein) group. The total actin protein fraction was lower than Con (9 ± 1 AU/μg protein) in the SI-C1 and SI-Stim1 groups (7 AU/μg protein), and similar in the SI-C2 and SI-Stim2 (9 AU/μg protein) groups. Compared with the contralateral controls, stimulation had no effect on the total MHC or actin protein fractions (Fig. 5B). The total MHC and actin protein content in all SI groups (~1,970 and ~630 AU/muscle in SI-C1 and ~1,630 and ~600 AU/muscle in SI-C2 groups, respectively) were lower than in the Con group (~4,310 and ~1,460 AU/muscle, respectively). Stimulation resulted in a 25 and 20% higher total MHC and actin protein content in the SI-Stim1, and a 53% higher total MHC protein content and a 39% higher actin protein content (P > 0.05) in the SI-Stim2 group, compared with their respective contralateral SI-C groups. In general, these data show that both the MHC and actin contractile proteins are responsive to the stimulation countermeasure.

Mean DNA concentration was higher than Con (1.22 ± 0.03 mg/g) in the SI-C1, SI-C2, and SI-Stim1 groups (1.57 ± 0.07, 1.53 ± 0.07, and 1.38 ± 0.04 mg/g, respectively), and it was similar in the SI-Stim2 (1.31 ± 0.05 mg/g) group. Mean DNA content was lower in all SI groups (631 ± 40, 526 ± 27, 615 ± 32, and 543 ± 20 μg/muscle in the SI-C1, SI-C2, SI-Stim1 and SI-Stim2 groups, respectively) compared with Con (837 ± 26 mg/g). This resulted in a 12% lower DNA concentration in the SI-Stim1 compared with the SI-C1 group (Fig. 5C). Stimulation had no effect on the mean DNA content compared with the SI-C groups. The protein-to-DNA ratio was lower than Con (0.19 ± 0 mg/μg), in the SI-C1, SI-C2, and SI-Stim1 (~0.15 mg/μg) groups, and similar in the SI-Stim2 (0.17 ± 0.01 mg/μg) group. This ratio was 17% higher in the SI-Stim1 compared with the SI-C1 group.

SDS-PAGE gels. The MG, a predominantly fast ankle extensor, contained ~9, 7, 35, and 49% types I, IIA, IIX, and IIB MHC isoforms, respectively, in the Con group (Fig. 6). After 30 days of SI, there was a shift in the MHC profile toward the fastest isoform, i.e., the MG of both groups of SI-C rats contained ~1, 1, 21 and 77% types I, IIA, IIX, and IIB MHC isoforms, respectively. In both SI-Stim groups, the percent type IIB MHC in the MG was similar to Con and lower than in the nonstimulated muscles. In contrast, the percent type IIX MHC of the SI-Stim1 and SI-Stim2 groups was ~32 and 40% higher than in the Con group, and it was 105 and 141% higher than in the SI-C1 and SI-C2 groups, respectively. In addition, the percent type I MHC in the SI-Stim2 group was higher than in the SI-C2 and SI-Stim1 groups, but lower than in the Con group.

Fiber-type composition. The MGd of Con rats was composed of 23, 3, 62, and 12% types I, I, IIX, and IIB fibers (Fig. 7A). In all SI groups, the percentage of type I fibers decreased to ~9% and the percentage of type I+IIX fibers increased to ~13%. In the SI-C1 and SI-C2 groups, the percentage of type IIX fibers was lower and that of the type IIB+ fibers higher than in the Con and SI-Stim groups. With both stimulation protocols, the percentage of types IIX and IIB+ fibers were not significantly different from Con levels.

The MGs region in all groups was composed of fibers containing only the fast MHC isoforms, with the Con group having 19% IIX and 81% IIB+ fibers (Fig. 7B). Inactivity resulted in a decrease (from 19 to ~10%) in the percentage of IIX fibers and a concomitant increase in the percentage of type IIB+ fibers (from 81 to ~90%). The fiber-type composition of the MGs region in both SI-Stim groups was similar to Con. Developmental MHCs were not detected in any fibers of any group.

Fiber-size adaptations. The mean CSA of all fiber types in the MGd region of Con rats ranged from ~1,500 to 2,500 μm² (Fig. 8A). Chronic inactivity resulted in a decrease in the mean size of all fiber types, with the relative effect being the greatest...
for the type I fibers. The range of mean fiber CSAs decreased to ~200 to 1,300 μm² in both the SI-C1 and SI-C2 groups. In general, the size of each fiber type was larger in the stimulated than the nonstimulated muscles, with the differences being significantly larger for all types in the SI-Stim2 group, but only for types I and I+IIa/x for the SI-Stim1 group. The fiber CSAs ranged from ~400 to 1,300 μm² in the SI-Stim1 group and from ~800 to 1,600 μm² in the SI-Stim2 group. Specifically, the I+IIa/x and IIa/x fibers were larger (95 and 32%, respectively) in the SI-Stim2 than SI-Stim1 group. The mean overall fiber CSA was ~36% larger in the SI-Stim2 than the SI-Stim1 group.

The mean CSAs of IIa/x and IIb+ fibers in the MGs region in the Con group were ~1,900 and 3,000 μm², respectively (Fig. 8B). Inactivity resulted in atrophy of the IIa/x and IIb+ fibers in both the SI-C1 (to ~700 and 1,300 μm², respectively) and SI-C2 (to ~800 and 1,500 μm², respectively) groups. The stimulation protocols attenuated the inactivity-induced atrophy: the mean CSAs were ~900 and 1,200 μm² for IIa/x fibers and ~1,700 and 2,000 μm² for IIb+ fibers in the SI-Stim1 and SI-Stim2 groups, respectively. The overall CSA of all fibers in the MGs region was ~25% larger in the SI-Stim2 than the SI-Stim1 group.

Relative contribution of each fiber type to the whole muscle CSA. The MGd was 33, 34, 29, 23, and 27% of the total muscle cross section in the Con, SI-C1, SI-C2, SI-Stim1, and SI-Stim2 groups, respectively. The relative contribution of each fiber type to the whole muscle cross section was determined for each group (Fig. 9). The relative contribution of pure type I fibers was lower in all SI groups (~1%) than in the Con group (8%). The contribution of type I+IIa/x fibers was ~1 to 2% in all groups. The percent contribution of type IIa/x fibers was lower in the SI-C (~18%) than in the Con (29%) and similar to Con in the SI-Stim (~24%) groups. The relative contribution of type IIb+ fibers was higher than Con in all SI groups, comprising 63% of the muscle cross section in the Con, ~80% in the SI-C, and ~70% in the SI-Stim groups.

DISCUSSION

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second for 30 min, delivered 5 days/wk, for 4 mo (37). The activity duration in that study totaled 9 min/day compared with the ~3.5–5.5 h observed in ambulatory cat soleus muscles (1, 25). The PCSA, Po, Vmax, and the percentage of type I MHC were maintained closer to control values in the soleus muscles that were stimulated isometrically compared with stimulated during the shortening or lengthening phase of a simulated step cycle (37). The effects of isovelocity and isometric contractions also were compared in the soleus, MG, and plantaris (Plt) muscle of hindlimb-unloaded rats stimulated at 100 Hz every other day for 4 wk (14). The mass of the fast MG and Plt muscles was unaffected by training, whereas both contraction protocols partially blunted atrophy of the slow soleus muscle. Furthermore, the isometric protocol had a positive effect on partially blunting the unloading-induced decrease in type I and increase in type Ila MHC in the soleus muscle. A similar muscle specific response was seen in a 7- and 14-day hindlimb-unloading study in which the rat hindlimb was stimulated with 10 5-s maximal isometric contractions every 90 s, for 3 sessions daily (23). Both muscle atrophy and fiber atrophy were attenuated in the soleus, but not in the MG or Plt, muscles. The soleus also showed an improved Pm. These stimulation paradigms presumably induced higher forces (higher frequency of activation and more muscle fibers activated) during any given activation compared with in vivo conditions.

Together, these studies demonstrate that 1) mechanical loading is a critical component in developing effective atrophy countermeasures; 2) isometric contractions are as, or more, efficacious than shortening and/or lengthening contractions for ameliorating the atrophy and phenotypic adaptations associated with inactivity or reduced activity; and 3) brief periods of neuromuscular activity, a fraction of the total time of some threshold level of activity observed in ambulatory control muscles, when delivered with the appropriate parameters, are sufficient to positively impact muscle properties. Furthermore, it is clear that an effective activity countermeasure for one muscle does not necessarily translate to other muscles, e.g., the predominantly slow soleus vs. the predominantly fast MG and Plt. A majority of previous electromechanical stimulation studies for blunting atrophy have resulted only in an activity response in the soleus muscle while having little to no effect on fast muscles. Therefore, one aim of the present study was to design an atrophy countermeasure for the fast MG muscle. We applied a high-load, short-duration isometric stimulation protocol daily to the atrophying MG in SI rats. The total activity duration was 1 min per day, i.e., only 0.22% of the ~7.5 h of activity normally observed in the MG of ambulatory control rats (22). These very short periods of high-load activity had a significant impact on the otherwise inactive MG, e.g., amelioration of ~25% of the muscle atrophy, ~28% of the loss in total protein and myofibril protein content, ~50% of the loss in maximum force production, and 100% of the loss in SpT.

A loss in SpT has been previously observed in the soleus of SI rats (39). SI of the soleus muscle was also associated with a significant loss of the MHC protein fraction, whereas the actin protein fraction relative to the total protein remained unchanged, resulting in an increase in the actin-to-MHC ratio (20). This disproportionate loss in the MHC protein fraction was not observed in the MG of SI-C groups. Results from the present study suggest that the actin-to-MHC ratio, along with the total protein and myofibrillar protein concentrations, do not account for the reduction in specific tension observed in the SI-C groups or for the subsequent maintenance at the normal range in the SI-Stim groups. The amelioration of total protein and myofibril protein content in the SI-Stim compared with the SI-C groups, however, are likely factors that contributed to both the improved mass and functional capacity of the MG to result in the maintenance of a normal SpT in the SI-Stim groups. In addition, stimulation training likely maintained the integrity of the muscle architecture to generate the normal amount of force per cross bridge.

An electromechanical activity protocol delivered in two vs. one session per day was more efficacious in maintaining muscle mass and functional capacity. Time course studies following unloading of the soleus muscle have demonstrated that atrophy is accompanied by a marked decrease in the rate of myofibrillar protein synthesis and an increase in the rate of myofibrillar protein degradation (45, 46). During this same time period, there is an initial decrease in the EMG activity of the unloaded soleus, which then returns to and increases above control levels by ~10 days of unloading (2), suggesting that the critical factor for regulating protein turnover is not the amount and level of activation (as measured by EMG activity) but the amount of loading produced during the contractile activity. The effects of a loaded contractile activity paradigm on protein turnover and muscle properties may be augmented by interspersing the stimulus over the course of the day to increase the time that the load induced influence on the protein turnover is positive. The present results clearly demonstrate that the blunting effect of 1 min of high-load electromechanical activity on the loss of mass, protein content, and functional capacity of the MG is further augmented by delivering the stimulation protocol in two vs. one session per day. Therefore, these data suggest that the number of sessions in which an electromechanical activation protocol is delivered over the course of the day is a critical factor in determining its efficaciousness as an atrophy countermeasure, suggesting that some minimum interval for activation is needed throughout the day to maintain homeostasis. It also should be noted that the stimulation paradigms used in the present study did not maintain muscle mass, and thus protein content, at control levels. Whether other stimulation regimes could be used to maintain muscle mass in inactive muscles at control levels is unknown.

Shifts in MHC phenotype are affected similarly with one or two sessions of electromechanical activity per day. A protective effect of mechanical loading on blunting the atrophy-associated slow to fast shift in MHC phenotype expression has been demonstrated in a number of studies (14, 37, 46). For example, a daily 30-min bout of isometric contractions (300 ms on and 700 ms off) was effective in blunting the type I to Ila MHC shift in the homogeneously slow soleus of adult SI cats (37). Similar concentric or eccentric contraction paradigms were much less effective. Isometric contractions also were more effective than isovelocity contractions in blunting MHC phenotype shifts in the soleus muscle in hindlimb-unloaded rats (14). All of these results are consistent with the relatively high percentage of “slower” fiber types and MHC isoforms in the MG of the limb being isometrically stimulated as in the present study. The blunting of the isoform shift from IIX to IIB MHC was observed with both stimulation protocols. These results suggest that the MHC phenotype properties of the MG...
are less dependent on a shorter interval between stimulation sessions than the properties related to the functional capacity and amount of total and myofibril protein in the muscle.

Relationship between MHC profile and $V_{\text{max}}$. Single fiber data have demonstrated a strong relationship between MHC isoform composition and $V_{\text{max}}$ (I $<$ IIA $<$ IIx $<$ IIb) (5, 7). Evidence also suggests that the fiber-type composition or MHC profile of a muscle is closely related to its force-velocity properties (9, 10, 31). These adaptations in speed properties are especially evident in the predominantly slow soleus muscle, which expresses only types I and IIA MHC isoforms. For example, in reduced activity models there is an increase in the expression of type IIA expressing fibers and a de novo expression of type IIX expressing fibers in the soleus muscle. This slow-to-fast shift in the MHC profile and de novo expression of faster isoforms are associated with an increase in $V_{\text{max}}$ (8, 37, 44). It is important to note, however, that a high degree of variability and overlap in the velocity of unloaded shortening values for fiber types have been reported. For example, Botti- 

neli et al. (5) reported that the range in speed properties of type IIX ($\sim$2.2–4.3 fiber lengths/s) and IIb ($\sim$1.5–6.0 fiber lengths/s) fibers almost completely overlap. Therefore, as might be expected, the SI-induced shift from IIX to IIb expressing fibers in the MG did not significantly affect $V_{\text{max}}$. These results are consistent with previously published measurements of atrophying fast muscles (13, 23).

In summary, the present results demonstrate that brief periods (a total of 1 min or 0.069% of a 24-h period) of patterned high-load, short-duration isometric contractions are sufficient to significantly reduce the inactivity-induced atrophic, phenotypic, and physiological adaptations in a fast rat hindlimb extensor muscle. Furthermore, the same stimulation protocol delivered in two vs. one session per day proved to be more efficacious for preserving the mass, fiber CSA, and $P_{\text{0}}$ of the MG muscle. Because the total number of contractions and duration of activation of both training protocols were identical, the differences in the response of the muscle to the two protocols are a direct result of the number of sessions of activation per day. Phenotype expression (of the fastest MHC isoforms) and SpT were maintained at control levels with both electromechanical stimulation protocols, demonstrating varying sensitivities of different muscle properties to loading protocols. These results suggest that a careful and thorough examination of the level and temporal patterns of activation of skeletal muscle relative to the level of maintenance of normal muscle properties is needed in developing rehabilitative strategies for regaining and preventing a loss of skeletal muscle function.

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