Passive stretch inhibits central corelike lesion formation in the soleus muscles of hindlimb-suspended unloaded rats

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Baewer, D. V., M. Hoffman, J. G. Romatowski, J. L. W. Bain, R. H. Fitts, and D. A. Riley. Passive stretch inhibits central corelike lesion formation in the soleus muscles of hindlimb-suspended unloaded rats. J Appl Physiol 97: 930–934, 2004.—Hindlimb suspension unloading (HSU) of rats is a well-established experimental model for simulating the effects of microgravity unloading on the musculoskeletal system. In this model, gravity causes the hind foot of the rat to drop, opening the front of the ankle to 90–105° planar flexion at rest. As HSU proceeds, the normal weight-bearing angle of 30° dorsiflexion is achieved progressively less, and the contraction range of soleus is abbreviated. Our laboratory reported that 12 days of HSU caused central corelike lesions (CCLs) of myofibril breakdown (Riley DA, Slocum GR, Bain JL, Sedlak FR, Sowa TE, and Mellender JW. J Appl Physiol. 69: 58–66, 1990). The present study investigated whether daily stretch of the calf muscles prevents CCL formation. The soleus muscles of HSU Sprague-Dawley male rats (~287 g) were lengthened by unilateral ankle splinting at 30°. Compared with the nonsplinted side, splinting for 10 or 20 min per day in awake rats significantly decreased CCLs in soleus by 88 and 91%, respectively (P < 0.01). Compared with control muscle wet weight, 20-min splinting reduced atrophy by 33%, whereas 10-min splinting ameliorated atrophy by 17% (P < 0.01). Bilateral soleus electromyograph recording revealed higher levels of contractile activity on the splinted side during splinting. To isolate the effects of stretch from isometric contractile activity, contractions were eliminated by whole animal anesthetia with isoflurane during 10-min daily splinting. The percentage of fibers with CCLs was reduced by 57%, and the average lesion size was 29% smaller in the stretched muscle (P < 0.05). Soleus muscle wet weight and fiber area were unaltered by stretch alone. Loaded contractions during splinting are necessary to prevent muscle fiber atrophy. Passive muscle stretch acts to maintain myofibril structural integrity.

METHODS

Hindlimb suspension unloading (HSU) of rats is a well-established experimental model for simulating the effects of microgravity unloading on weight-bearing muscles (25). Preventing rats from touching the ground with their hindlimbs causes the antigravity soleus muscles to atrophy due to decreased use, unloading, and shortened working range (29). Central corelike lesions (CCLs), which are areas of focal myofibril dissolution, appear within soleus fibers by day 12 of HSU. For rat solei, the CCLs were postulated to result from the ~20% shortened contraction range, which is a consequence of suspension-induced foot drop posture (plantar flexion) (22, 29). Foot drop posture of humans floating in microgravity was first documented in their neutral body position during Skylab missions (26). CCLs also occur in suspension-unloaded rabbits in which soleus shortening is ~35% because the large hind feet are weighed down by gravity (2). A shortened length is required for CCL generation because the HSU rabbits do not exhibit CCLs when their hind feet are supported with elastic bands to prevent foot drop (30). The elastic bands provided resistance, albeit small, and these loaded contractions may have contributed to prevention of lesions. CCLs occur in other conditions of chronic shortening, such as tenotomy and immobilization by casting in a shortened position (4, 8, 19). The degeneration of myofibrils after tenotomy correlates with the hypershortened state of the sarcomeres (3). The formation of CCLs in tenotomy is inhibited by denervation, suggesting that contractile activity is essential for lesion generation (1, 34). It is unclear how muscle shortening and contractile activity interact to generate CCLs. The objective of the present study was to investigate the role of muscle length, with and without contractile activity, on myofibril structural integrity in HSU rats.

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intermittently weight-bearing HSU rats, we observed that CCLs were dramatically reduced (Riley, unpublished observations). Splinting at a constant angle was employed rather than stretching by standing because standing was subject to uncontrolled variability due to ad libitum movement of the rats.

**Tissue processing.** After completion of 12-day HSU, rats were weighed when deeply anesthetized with a ketamine (72 mg/kg), xylazine (12 mg/kg), and acepromazine (0.09 mg/kg) mixture delivered intramuscularly. Soleus muscles were excised by transecting the sciatic nerves (Figs. 2, B and C, and 3, A and B). Digital microscopic images of the acid-preincubated ATPase sections were taken with a SPOT II charge-coupled device camera (model 1.40, Diagnostic Instruments) mounted on a Nikon Eclipse E600 microscope. For muscle fiber sampling a standardized rectangle (400 × 370 μm) was placed in each of four quadrants of the section image (×200) in an area free of artifacts and large blood vessels. The number of fibers per section with CCLs was counted and expressed as a percentage of the total fibers sampled in the section with an average of 140 ± 20 fibers sampled per section. For the splinted ISF-anesthetized group, average CCL size per lesioned fiber was measured with Metamorph 4.5 software by thresholding the lesioned regions within a fiber to obtain the percentage of fiber area occupied by the lesion. Thresholding was performed for each fiber 10 separate times and averaged to increase the reliability of the measurement. The thresholded lesion area divided by the total fiber area generated the percentage of lesioned area.

To determine muscle fiber size, the cross-sectional areas (CSFA) of 50 muscle fibers residing totally within each of the 4 sampling rectangles were measured. For each muscle, 200 fibers were traced by digitizing planimetry along the perimeter, and their areas were calculated by Metamorph 4.5 software. Statistical analysis of the data was done with ANOVA, post hoc, Tukey, and t-test as appropriate.

**Monitoring muscle contractile activity during splinting.** Two HSU rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and instrumented bilaterally with epimysial EMG electrodes for recording soleus muscle activity. The electrode implantation procedure is described in detail by Hurst and Fitts (16). When the splints were put on these awake rats, they vigorously pushed against the splint. EMG activity was noticeably higher on the splinted compared with the nonsplinted side. The contracting triceps surae muscles felt firm when palpated. To determine whether increased EMG activity occurred each time the leg was splinted for 10 min daily during 12 days of HSU, monitoring of soleus muscle activity was performed in the two rats by simultaneously recording from the splinted and nonsplinted sides starting 15 min before and continuing until 15 min after the splinting procedure. The EMG signals were digitized at 2,000-Hz sampling rate and processed by Spike 2 software to assess integrated EMG activity (mV/s) and average train duration (s). Trains were defined as seven consecutive spikes no greater than 19 ms apart. For each rat, activities were compared between the two sides.

**Elimination of voluntary muscle contractions during splinting.** Having observed robust isometric contractile activity in soleus muscles of the splinted legs of awake HSU rats, a group of HSU rats was anesthetized while suspended before the splinting session to test the effects of lengthening (stretching) muscles in the absence of contractile activity. Rats were deeply anesthetized with ISF gas (Drager

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**Fig. 1.** Lengthening of the soleus muscle by ankle splinting. The ankle splint is slid onto the leg and foot from the back and held in place by a rubber band strap stretched across the front of the ankle and around the splint. The ankle is held at 30°, and the soleus muscle is stretched.

**Fig. 2.** Soleus muscle fiber sections from control and hindlimb-suspended unloaded rats stained for myofibrillar ATPase after acid preincubation. A: control muscle fibers are the largest and exhibit uniform staining across all fibers. B: fibers in the 20-min splinted awake group from nonsplinted legs are the smallest, and they contain tightly staining foci characteristic of central corelike lesions (arrows). C: fibers in the splinted legs are smaller than control but larger than the nonsplinted, and few fibers exhibit central corelike lesions (arrows). Bar = 70 μm.
was expected for this size rat because they have been shown to decrease food intake initially before resuming weight gain (25). Weight decrease (24 g) of the ISF-anesthetized group was significantly greater \((P < 0.01)\) than that \((9 g)\) of the nonanesthetized, 20-min splinted group. On the basis of the ratio of muscle wet weight (MW) to body weight (BW), all HSU muscles were significantly smaller than controls \((P = 0.01)\). Compared with nonsuspended control muscles, solei in the 20-min awake and 10-min ISF splinted HSU groups atrophied on the basis of both CSFA \((\mu m^2)\) and MW \((mg)\) normalized to BW \((g)\) \(\text{Fig. 2, Table 1}\).

**Splitting of Awake HSU Rats.** Within the 10- and 20-min splinted, awake groups, the MW-to-BW ratios for solei on the splinted side were significantly larger than those of the nonsplinted side \((P = 0.01)\). The difference between the MW-to-BW ratios of the splinted and nonsplinted muscles was significantly greater for the 20-min splinted group compared with that of the ISF and 10-min splinted groups. CCLs were virtually absent \((<1\%)\) in control muscles \(\text{Fig. 2A}\). In the 10- and 20-min splinted awake groups, the splinted soleus exhibited a significantly lower percentage of CCLs than the nonsplinted side \(\text{Fig. 2, B and C, Table 1}\).

**Muscle contractile activity during splitting of awake HSU rats.** During the splitting session, integrated EMG activity was significantly \((P < 0.05)\) increased by 31\% in the splinted muscle relative to the nonsplinted side \(\text{Fig. 2B}\). Average train duration was dramatically prolonged by 50\% in the splinted soleus compared with the nonsplinted side \(\text{Fig. 2C}\).

**Splitting Anesthetized HSU Rats.** In the ISF-anesthetized rats, the leg muscles did not contract when the splint was applied. The mean percentage of fibers with CCL lesions was significantly lower on the splinted than the nonsplinted side.

### Table 1. Indexes of muscle atrophy and CCL occurrence in soleus muscles

<table>
<thead>
<tr>
<th>Group</th>
<th>MW-to-BW Ratio, mg/g</th>
<th>CSFA-to-BW Ratio, (\mu m^2/g)</th>
<th>Percentage CCL Fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ((6))</td>
<td>0.40 \pm 0.04</td>
<td>10.7 \pm 1.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>HSU awake ((5))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-min nonsplinted</td>
<td>0.19 \pm 0.04*</td>
<td>4.1 \pm 0.8</td>
<td>26.8 \pm 2.9</td>
</tr>
<tr>
<td>20-min splinted</td>
<td>0.26 \pm 0.03*†</td>
<td>7.3 \pm 1.4*‡</td>
<td>2.5 \pm 1.3‡</td>
</tr>
<tr>
<td>HSU awake ((4))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-min nonsplinted</td>
<td>0.22 \pm 0.01 *</td>
<td>ND</td>
<td>27.5 \pm 5.1</td>
</tr>
<tr>
<td>10-min splinted</td>
<td>0.25 \pm 0.02*‡</td>
<td>ND</td>
<td>3.3 \pm 1.2‡</td>
</tr>
<tr>
<td>HSU anesthetized ((6))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-min nonsplinted</td>
<td>0.25 \pm 0.01*</td>
<td>4.0 \pm 0.2</td>
<td>20.6 \pm 3.0</td>
</tr>
<tr>
<td>10-min splinted</td>
<td>0.24 \pm 0.01*</td>
<td>4.0 \pm 0.2</td>
<td>8.9 \pm 2.2†</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE; nos. in parentheses are no. of rats. Muscle wet weight \((MW)\) and cross-sectional fiber area \((CSFA)\) are normalized to body weight \((BW)\). HSU, hindlimb suspended unloaded; percentage CCL fibers, percentage of total fibers sampled that had central corelike lesions; ND, not determined. HSU significantly different from control, \(* P < 0.01, † P < 0.05\).

**RESULTS**

**Body and muscle weight changes.** After 12 days of HSU, the rats were lighter than their starting weights. Diminished weight...
The average lesion size (% of fiber area lesioned) in the splinted muscles (31.2 ± 7.5%) was significantly (P < 0.05) smaller than that in the nonsplinted muscles (43.7 ± 5.5%) (Fig. 3, A and B). The MW-to-BW and CSAF-to-BW ratios in the splinted and nonsplinted muscles were significantly smaller than control but not different from each other (Table 1).

**DISCUSSION**

**Effect of stretch on CCL formation and atrophy.** Soleus muscles of one leg of the HSU rats were subjected daily to sustained stretch, uniform in amount and duration, by applying an external splint fixing the ankle joint at 30° to simulate the quadrapedal standing angle. This controlled approach is an improvement over the model of intermittent standing weight bearing (6, 10) and tapping hindlimbs to hold them in a dorsal flexed position (23). Even though muscle atrophy was diminished by standing and tapping, determining quantitatively which aspects of the countermeasure impacted muscle mass was difficult because the degree of stretching and amount of active loading were uncontrolled. The effects of those countermeasures on the development of CCLs were not addressed. Loughna et al. (23) found no significant difference in the atrophy of HSU soleus muscles that were stretched by taping for 6 h per day vs. 24 h per day and found that 30 min per day was effective at reducing atrophy. In the present study, we determined that in awake HSU rats as little as 10 min of splinting was sufficient to significantly reduce CCL lesion occurrence and muscle atrophy after 12-day HSU. Atrophy inhibition exhibits a dose-dependent response to the duration of contractile activity. This is demonstrated by the intra-animal MW-to-BW differences, with the largest atrophy inhibition found in the longest duration splinting experiment (20 min).

The decreased length of the soleus during HSU, which is suspected to cause CCLs, was extended to the normal weight-bearing length during sustained stretch of the soleus by splinting. To distinguish between the roles of stretch and contractile activity, contractile activity was eliminated during lengthening by ISF anesthetizing the HSU rats during 10-min splinting. Under this condition, muscle atrophy was not prevented, but CCL occurrence was significantly reduced.

The magnitude of the CCL reduction in the anesthetized group was less remarkable than that in the nonanesthetized rats. This suggests that brief daily muscle lengthening (stretch) alone, in the absence of contractile activity, is able to counter the myofibril instability characterized morphologically by CCLs that develop in the shortened soleus muscles of HSU rats. Contrary to previous reports that claimed atrophy reduction due to passive stretch, we have shown that contractile activity is necessary for atrophy inhibition when stretched for 10 min per day (20).

**Is the generation of CCLs in HSU muscles similar in process to central core muscle disease?** The CCLs described for HSU are morphologically similar to the lesions found in human central core disease (CCD). Is there a similarity in the process of lesion formation? CCD is linked to gene mutations of the muscle-specific Ca$^{2+}$ release channel (ryanodine receptor 1). Current thinking is that these mutations generate leaky channels and result in excessive release of Ca$^{2+}$ from the sarcoplasmic reticulum. This release exceeds the capability of the cell to adequately regulate free Ca$^{2+}$ in the central region of the fiber (21, 24). Loke and MacLennan (21) have proposed that, in CCD fibers, Ca$^{2+}$ overload occurs in the center of the fiber, presumably too distant to be aided by peripheral homeostatic mechanisms. The central increase in Ca$^{2+}$ is suspected to generate CCLs.

The calcium-overload hypothesis for CCD with mutant ryanodine receptors may be relevant to CCL formation in HSU rats. Even though ryanodine receptors are genetically normal, disruption of calcium homeostasis occurs in the soleus muscles of HSU rodents. Intracellular resting calcium concentration increases progressively to twice normal in shortened soleus muscle fibers in HSU mice (17, 18). Elevated Ca$^{2+}$ activates calcium-activated proteases, calpains, which partially degrade myofibrillar proteins and ryanodine receptors, leading to breakdown of myofibrils and enhanced calcium efflux (11, 27, 31). Calcium-activated protease activity increases in atrophying soleus muscles during HSU (12, 33) and spaceflight (28) and directly parallels the development of CCLs in tenotomy (5). Immobilized soleus muscles show significant increases in total calcium concentration (7). The calcium channel blocker nifedipine markedly reduces immobilization atrophy and ultrastructural mitochondrial damage, possibly by protecting the muscle fibers from calcium overload (32). The findings in experimental models of chronically shortened active muscles suggest that the development of CCLs is the consequence of excess intracellular calcium. Experiments to examine this hypothesis are underway.

**How does brief stretch reduce CCL formation in HSU soleus muscles?** It is not understood how stretch counteracts CCL formation. Assuming a progressive central buildup of calcium, stretch may prevent calcium accumulation by reducing the overlap of thick and thin filaments, which lowers myofilament protein density and decreases fiber diameter. These physical changes would promote diffusion of calcium to the cell periphery for removal. Brief stretch is envisioned to reverse the progressive calcium buildup and restore calcium homeostasis, inhibiting calcium from exceeding the threshold point where calpains are activated to degrade myofilaments and ryanodine receptors.

Other explanations may account for the stretch-mediated stabilization of myofibrils. The mechanical stimulus of muscle fiber stretch is known to promote anabolic gene expression (14). Loughna et al. (23) showed that stretching HSU soleus muscles increases protein synthesis. Stretch-mediated production of myofibrillar proteins would counteract the development of CCLs. The inhibition of CCL formation by splinting was less in the ISF group, which seems likely due to a much stronger anabolic stimulus in the 10- and 20-min awake splinted groups, which actively contracted while splinted. Another possibility is that length responsive changes are signaled by potential mechanosensors such as obscurin, titin, focal adhesion kinase, and the dystrophin glycoprotein complex (9, 13, 36). The muscle-specific calpain 3 (p94) interacts with and cleaves titin, which may also function in stretch signaling (35). It is important to understand the mechanisms by which stretch and moving a muscle through its full working range promotes structural stability. We utilized anesthesia to eliminate contractile activity during splinting. This permitted us to conclude the existence of a solely stretch-induced signal that stabilizes myofibrils. Although the benefits of stretching on muscle...
health are well recognized in physical therapy and exercise, the mode of action is unknown (15). Understanding the cellular and molecular mechanisms by which stretch operates is important for clinical medicine, sports and exercise.

GRANTS

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