Supplementary Methods Section for Preliminary Study

Static stretching exercise

Rats were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg Somnopentyl®; Kyoritsu Seiyaku Co., Tokyo, Japan) and the bilateral soleus muscles were stretched with the custom-built apparatus described in the current study. The amplitude of static stretches was controlled with a stepping motor (linear motor LU4B45SA-2; Oriental Motor Co. Ltd., Tokyo, Japan). Stretching exercises were performed at the day's maximum dorsiflexion angle, as measured with a goniometer. The static stretching was applied for 30 min/day, 6 days/week, beginning immediately after cast removal (prior to reloading) and continuing for 1 or 2 weeks (6 or 12 sessions total).

Myosin ATPase staining

The rats were sacrificed with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) on Day 0, 7, or 14 after cast removal and the soleus muscles from both hindlimbs of each rat were excised. Soleus muscles were embedded in optimal cutting temperature compound (TissueTek®; Sakura Finetek, Tokyo, Japan); 7-µm cross-sections were cut from the mid-portion of the muscles with a cryostat (CM1510-11; Leica, Wetzlar, Germany) and mounted on Superfrost Plus slides (Thermo Fisher Scientific, Tokyo, Japan).

Myosin ATPase staining was performed according to the protocol of Brooke and Kaiser (1970). Briefly, sections were pre-incubated in acidic buffer (0.1 M barbital acetate and 0.1 M hydrochloride, adjusted to pH 4.6) for 5 min and then rinsed with a substrate solution (0.18 M calcium chloride and 0.1 M sodium barbital, adjusted to pH 9.4). Sections were then incubated in ATP staining buffer (0.18 M calcium chloride, 0.1 M sodium barbital, and 2.4 mM ATP disodium salt) at pH 9.4 for 45 min, washed three times in 1 % calcium chloride solution for 3 min each, incubated with 2 % cobalt chloride for 3 min, washed eight times with 0.01 M sodium barbital solution, and rinsed with distilled water for 2 min. Finally, sections were incubated in 1 % ammonium sulfide for 1 min and rinsed with distilled water five times. Following staining, each section was sealed with Canada balsam and topped with a coverslip. Dark-stained fibers were classified as Type I (slow fibers) and light fibers as Type II (fast fibers). Type IIA fibers appeared white, whereas type IIB fibers stained gray (Lind and Kernell 1991).

Images of the stained cross-sections were captured with an optical microscope (BZ-9000; Keyence, Osaka, Japan) at 20× magnification. The cross-sectional area of each fiber type in the soleus muscles was measured with Image J software (National Institutes of Health, Bethesda, MD, USA). More than 100 fiber measurements were recorded per animal for each type of fiber.

References

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