

# Effects of Eccentric Exercise on Joint Stiffness and Muscle Connectin (Titin) Isoform in the Rat Hindlimb

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**Abstract:** We investigated the effects of repeated eccentric exercise for rat medial gastrocnemius muscle on ankle joint stiffness and muscle connectin (titin) isoform composition (longer form,  $\alpha$ -connectin; shorter form,  $\beta$ -connectin). Male Wistar rats were trained on a custom-made, isokinetic dynamometer (eccentric-exercise group,  $n = 6$ ; sham-operated group,  $n = 6$ ). The exercise session consisted of 20 eccentric contractions elicited by submaximal electric stimulations under anesthesia. The contracting muscle was forcibly lengthened by an isokinetic dorsiflexion of the ankle joint (velocity, 30°/s; range of motion, 45°). Rats in the eccentric-exercise group were trained every two days for 20 days (10 sessions in total). The static passive resistive torque (PRT) of 45° at the ankle joint was used as a mea-

sure of the joint stiffness, and was determined before and after the experimental period. After 10 sessions of eccentric exercise, the wet weight of medial gastrocnemius muscle significantly increased ( $P < 0.05$ ), whereas the static PRT significantly decreased ( $P < 0.05$ ) in the eccentric-exercise group, when compared to the sham-operated group. Myosin-ATPase staining showed a decrease in the number of type IIb/IIc fibers ( $P < 0.001$ ) and an increase in the number of type IIa fibers ( $P < 0.05$ ). However, no significant difference was seen in the connectin (titin) isoform composition between the eccentric-exercise group and the sham-operated group, suggesting that the reduction in PRT was not due to change in resting mechanical properties of muscle fibers.

**Key words:** muscular hypertrophy, joint stiffness, eccentric exercise, muscle fiber type, connectin.

Appropriate joint stiffness is regarded as important to prevent injuries and to improve physical activities [1–3]. The joint stiffness is generally defined as an absolute torque that is required to maintain a particular joint angle or change in the joint torque against the change in joint angle [4]. It likely involves the following three components: (i) elastic properties of connective tissues, including joint capsule and skin, (ii) elastic properties of the muscle-tendon complex, and (iii) neuromuscular reflex against stretch.

It has been shown that joint stiffness is affected by exercise. Previous studies have demonstrated that one bout of eccentric exercise for untrained human muscle caused an increase in joint stiffness [5–9]. For instance, the range of motion (ROM) of elbow joint decreases immediately after repeated eccentric contractions of elbow flexor muscles, and the decrease persists for 2–3 days [5, 6]. Joint stiffness evaluated as passive resistive torque (PRT) or passive elastic stiffness [7–9] has also been shown to increase after a bout of strenuous eccentric exercise. The post-exercise increase in joint stiffness may be caused by oedema [6, 8], a remodeling of connective tissues [10], or an imbalance of calcium homeostasis in the damaged fi-

bers [5], but not by persistent neuromuscular activity [11].

On the other hand, the chronic effects of eccentric exercise on joint stiffness are still unknown. It has been reported that increased elbow joint stiffness after a bout of eccentric exercise returned to its original level after three bouts of the exercise performed on three separate days [12]. However, it remains unclear how eccentric exercise for a longer period, such as that causing changes in muscular size and strength, affects joint stiffness.

While an appropriate animal model is greatly useful to study both the macroscopic and microscopic effects of exercise on joint function, only a rat hindlimb-suspension model has been used so far [13]. It has been reported that the static PRT of rat ankle joint significantly increased after a 7-day hindlimb suspension, and the increased joint stiffness was due mainly to an increase in the stiffness of muscle-tendon complex [13]. The parallel elasticity of muscle-tendon complex involves both extracellular connective tissue and a cytoskeletal protein connectin (titin), which has been reported to generate 41.3% of total passive tension in gastrocnemius muscle fibers [14]. Connectin is a gigantic (2.5–3.5 MDa) and unique protein that directly links the Z-disc and M-line. The I-band region of

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connectin functions as a molecular spring that develops passive tension when sarcomeres are stretched [15, 16]. Connectin often shows two major bands when analyzed with gel electrophoresis [17]. The band with larger molecular weight is defined as either alpha-connectin or T1 (intact connectin) and the band with lower molecular weight is defined as beta-connectin or T2 (degradation product of intact connectin) [18, 19]. Several studies have investigated the relationship between resistance training and connectin isoform composition in human muscle. Although it has been shown that, in humans, exercise training did not cause a considerable change in the muscle connectin isoform composition [20], it has also been shown that  $\beta$ -connectin isoform percentage tends to be lower in competitive athletes than in non-athletes [21]. Therefore, a prolonged period of strenuous exercise training may cause any change in  $\beta$ -connectin, thereby affecting joint stiffness.

In the present study, we investigated the effects of repeated eccentric exercise for the rat medial gastrocnemius muscle on the ankle joint stiffness and muscle connectin isoform composition. We hypothesized that sessions of eccentric exercise cause increases not only in muscular size and strength, but also in joint stiffness and a decrease in  $\beta$ -connectin isoform.

## METHODS

**Animals.** Male Wistar rats (age, 12 wk; body weight, 280–350 g;  $n = 12$ ) were randomly assigned into two groups: the eccentric-exercise group ( $n = 6$ ) and the sham-operated group ( $n = 6$ ). They were housed individually in a steel case maintained at 22–24°C and a 12-h light/dark cycle. In both groups, the hair of the right hindlimb was shaved and the animals were then anesthetized with isoflurane for the torque measurement and exercise. They took water and food *ad libitum*, and no significant differences were found between the groups in age and weight. The study was approved by the Ethical Committee for Animal Experiments at the University of Tokyo.

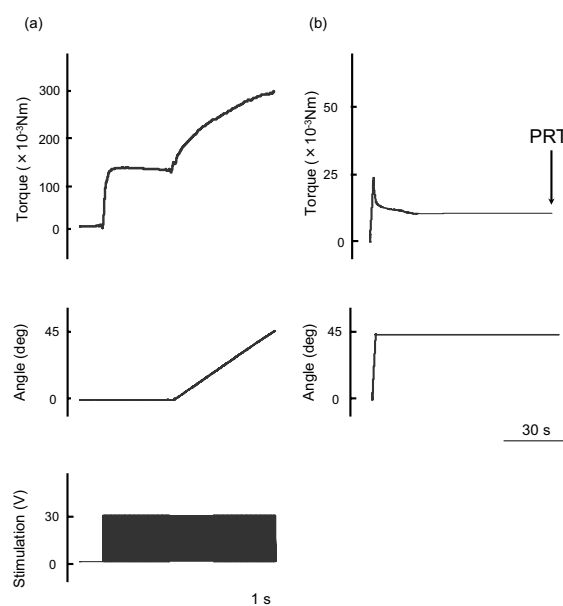
**Isokinetic dynamometer.** The ankle torque was measured on a dynamometer, which was developed according to Song *et al.* [22]. A footplate was driven by a stepping motor (RKD514HA, Oriental Motor, Japan) at varied angular velocities with a 5°/s step. The final deceleration and backlash movement of the footplate were damped by using a magnetic powder brake (ZKB-0.3AN, Mitsubishi Electric, Japan).

The footplate was positioned so that the anatomical axis of the ankle coincided with the axis of the dynamometer shaft. The plantar flexion torque was measured with a strain-gauge force transducer (LTB-2KA, Kyowa Electronic Instruments, Japan) attached to the footplate. The angular position of the dynamometer shaft was measured with a potentiometer (LP06M3R1HA, Murata Manufac-

turing, Japan). Both torque and position signals were sampled at 4,000 Hz by using a data acquisition system (PowerLab/16SP, ADInstruments, Australia).

**Measurement of isometric tetanic torque.** The isometric plantar flexion torque was measured with the dynamometer at the ankle joint angle of 0° (see below for the determination of ankle joint angle). Before measurement, the hairs of the right hindlimb were shaved for electrical stimulation. Anesthetized rats were laid prone on a platform and the knee was kept extended. The triceps muscle was stimulated supramaximally (pulse duration 0.4 ms; frequency, 100 Hz; intensity, ~35 V) through a surface electrode (7.5 mm  $\times$  7.5 mm) connected to the electric stimulator and isolator (Nihon Koden, Japan). Measurements were made before and after the exercise training period.

**Eccentric exercise for triceps muscle.** Rats in the eccentric-exercise group were trained every two days for 20 days (10 sessions in total) after measurements of the isometric tetanic torque. The rats were anesthetized with isoflurane, firmly fixed on a platform of the isokinetic dynamometer in a prone position, and the triceps surae muscle of the right hindlimb was electrically stimulated as previously mentioned. The stimulus voltage was adjusted to produce submaximal isometric twitch torque. The muscle was then stimulated for 3 s to cause submaximal tetanic contraction. One second after the onset of stimulation, a forced isokinetic dorsi-flexion was given to the ankle joint to cause an eccentric contraction of the triceps surae muscle (Fig. 1a). The speed and the range of forced lengthening were 30°/s and from 0 to 45°, respectively. The exercise session consisted of four sets of 5 contractions. The interval between each set was 5 min. This protocol for the



**Fig. 1.** Typical records of eccentric contraction (a) and static passive stretch (PRT) (b). The static passive resistive torque (PRT) was determined as the value of torque remained after stress relaxation was allowed for 90 s.

eccentric exercise was based on some previous studies [22–24] and our preliminary experiments. Only the measurements of isometric tetanic torque were made for the sham-operated group after they were anesthetized and shaved as the animals in the exercise group were. Within 12 h after the last exercise, the muscle specimens were dissected, weighed, and immediately frozen in liquid N<sub>2</sub> and stored at –80°C until analysis.

**Passive resistive torque against ankle dorsi-flexion.** The static passive resistive torque (PRT) of the ankle joint against dorsi-flexion was measured with the dynamometer to evaluate joint stiffness [13]. The ankle joint of anesthetized rat was dorsi-flexed from 0° (defined as the angle at which the sole of the foot and tibial bone are orthogonally positioned) to 45° at an angular velocity of 30°/s. A joint angle of 45° was chosen because it was shown as optimal for plantar flexion torque generation by our preliminary experiment. After the dorsi-flexion was completed, the stress relaxation was allowed to proceed for >90 s until the passive torque reached an almost steady level (Fig. 1b). The values of torque measured 90 s after the stretch were used as static PRTs.

**Myosin ATPase histochemistry.** A part of the frozen sample was subjected to myosin ATPase histochemistry. Before staining, the muscle sample was mounted on a Tissue Tek crytome and sectioned at 10 µm thickness at –20°C. Sections from each group were placed on the same glass slide to equalize the staining condition. The procedure for myosin ATPase staining was modified from Lind and Kernel [25]. Fiber types were distinguished by an 8-

min preincubation at pH 4.45 (Fig. 2). After staining, the fibers were observed under the light microscope and classified into slow type I, fast type IIa, and fast type IIb or IIc (type IIb/d). A cross-sectional area of muscle fibers was determined by using NIH image (version 1.61; National Institutes of Health, Bethesda, MD). For each rat, a minimum of 250 fibers were examined. To determine fiber-type distribution, five visual fields were analyzed for one muscle specimen (1,800 fibers in total).

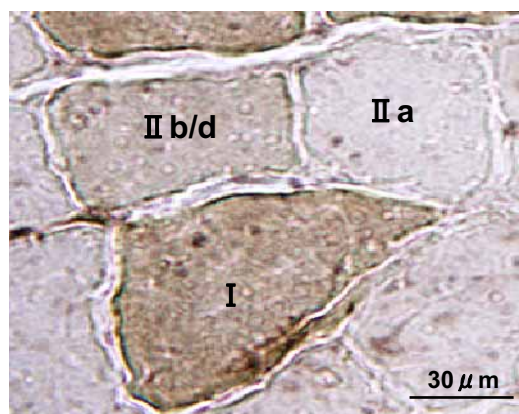
**Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE).** Frozen tissue samples were homogenized in 8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM DTT, 0.05 M Tris-Cl (pH 6.8) with a polytron homogenizer (KINEMATICA AG, Littau, Switzerland). The homogenate was heated at 80°C for 10 min, vortexed, and subsequently centrifuged for 5 min at 10,000 × g. The supernatant was analyzed with SDS-PAGE either immediately or after storage at –80°C. Low percentage SDS-PAGE was employed to distinguish connectin isoform: agarose-strengthened 2.0% polyacrylamide gel with a Laemmli buffer system without stacking gel [17]. Protein bands were visualized with Coomassie Brilliant Blue R250 and analyzed for optical density by using Light Capture densitometer (ATTO, Tokyo, Japan). To distinguish as reference α- and β-connectin, we loaded samples from rat heart on the same gels [14]. Due to this procedure, only four samples could be analyzed simultaneously for each group (see Fig. 5).

**Statistical analysis.** All values are expressed as means and standard deviations. Student's *t*-test was used to compare the body weight, muscle wet weight, muscle wet weight per body weight, the CSA of muscle fibers, fiber type composition, and the content of β-connectin between eccentric-exercise and sham-operated groups. Two-way analysis of variance (ANOVA) followed by Tukey's HSD was used for isometric tetanic torque and static PRT (time course × groups). Pearson's product-moment correlation coefficient was used to assess the relationship between the static PRT and the muscle wet weight. Significant level was set at *P* < 0.05.

## RESULTS

### Changes in muscle weight and fiber CSA

Both wet weight and wet weight/body weight of medial gastrocnemius muscle in the eccentric-exercise group



**Fig. 2.** Fiber-type determination by myosin-ATPase staining (pH 4.45). Dark, medium, and light stainings indicate type I, IIb/d, and IIa, respectively.

**Table 1.** Whole body weight, muscle wet weight, and muscle wet weight per body weight.

	Sham-operated group ( <i>n</i> = 6)	Eccentric-exercise group ( <i>n</i> = 6)
Whole body weight (g)	349 ± 10.2	350 ± 10.7
Wet weight of medial gastrocnemius muscle (mg)	731 ± 48.0*	815 ± 54.4*
Wet weight of medial gastrocnemius muscle/body weight (mg/g)	2.10 ± 0.161*	2.33 ± 0.184*

\**P* < 0.05. Values are means ± SD.

**Table 2.** Changes in isometric tetanic torque and static PRT.

	Before exercise	After exercise
Isometric tetanic torque ( $\times 10^{-3}$ Nm)		
Sham-operated group ( $n = 6$ )	213 $\pm$ 34.8	231 $\pm$ 27.3 <sup>†</sup>
Eccentric-exercise group ( $n = 6$ )	226 $\pm$ 24.9***	282 $\pm$ 34.5*** <sup>†</sup>
Static PRT ( $\times 10^{-3}$ Nm)		
Sham-operated group ( $n = 6$ )	8.42 $\pm$ 2.05	9.48 $\pm$ 3.12 <sup>†</sup>
Eccentric-exercise group ( $n = 6$ )	9.71 $\pm$ 2.78***	5.08 $\pm$ 1.65*** <sup>†</sup>
Static PRT/body weight ( $\times 10^{-3}$ Nm/kg)		
Sham-operated group ( $n = 6$ )	24.0 $\pm$ 6.07	27.1 $\pm$ 9.39 <sup>†</sup>
Eccentric-exercise group ( $n = 6$ )	27.8 $\pm$ 8.10***	14.5 $\pm$ 4.49*** <sup>†</sup>
Static PRT/muscle wet weight ( $\times 10^{-3}$ Nm/g)		
Sham-operated group ( $n = 6$ )	10.3 $\pm$ 2.00	11.5 $\pm$ 3.08 <sup>†</sup>
Eccentric-exercise group ( $n = 6$ )	13.3 $\pm$ 3.55***	6.89 $\pm$ 1.97*** <sup>†</sup>

Values are means  $\pm$  SD. \*\*\* $P < 0.001$ , before exercise vs. after exercise. <sup>†</sup> $P < 0.05$ , eccentric-exercise group vs. sham-control group.

**Table 3.** Correlation between wet weight of medial gastrocnemius muscle and static PRT.

	$r$	$P$ -value
Sham-operated group ( $n = 6$ )	0.820	0.045
Eccentric-exercise group ( $n = 6$ )	0.587	0.221

were significantly larger than those in the sham-operated group (wet weight,  $P = 0.018$ ; wet weight/body weight,  $P = 0.043$ ), even though no significant differences were seen for the lateral gastrocnemius and soleus muscles (Table 1). In the medial gastrocnemius muscle, the mean CSA of type IIa fibers was also significantly larger in the eccentric-exercise group than in the sham-operated group ( $P = 0.043$ ; Fig. 3).

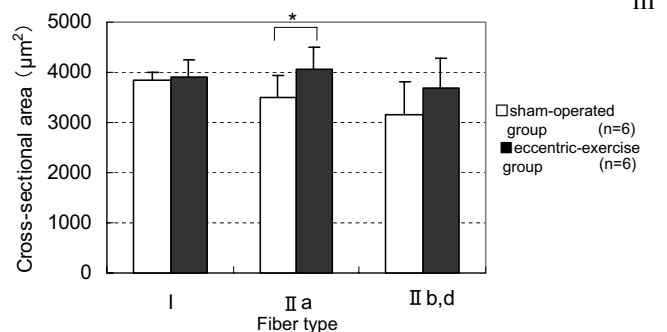
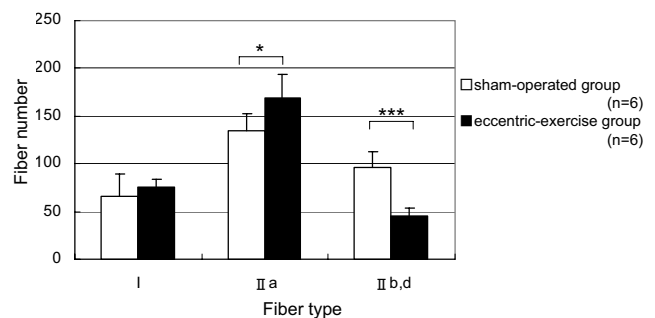
### Changes in active and passive joint torque

Table 2 summarizes changes in isometric tetanic torque and static PRT in the eccentric-exercise and sham-operated groups. Before eccentric exercise, the isometric tetanic torque in the eccentric-exercise group was  $226 \pm 24.9 \times 10^{-3}$  Nm and similar to that in the sham-operated group ( $213 \pm 34.8 \times 10^{-3}$  Nm). After 10 sessions of eccentric exercise, it showed a significant increase ( $P = 0.001$ ) from the pre-training value in the eccentric-exercise group and was also significantly larger than in the sham-operated group ( $P = 0.018$ ).

On the other hand, the static PRT in the eccentric-exercise group showed a significant decrease when compared with the pre-training value ( $P = 0.001$ ) and was also significantly smaller than that in the sham-operated group ( $P = 0.012$ ). For both isometric tetanic torque and PRT, the sham-operated group did not show significant changes from pre-exercise values. Similar results were obtained when PRT was normalized with either body weight or muscle weight.

### Correlation between passive resistive torque and muscle wet weight

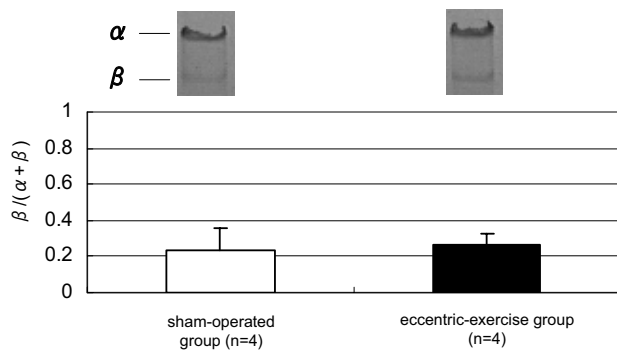
Correlation between static PRT and the wet weight of

**Fig. 3.** Cross-sectional area (CSA) of muscle fibers in the medial gastrocnemius muscle after a 20-day eccentric exercise. Values are means  $\pm$  SD. \* $P < 0.05$ .**Fig. 4.** Fiber type composition in the medial gastrocnemius muscle after a 20-day eccentric exercise. Values are means  $\pm$  SD. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

medial gastrocnemius muscle was examined for specimens taken after the period of eccentric exercise (Table 3). The sham-operated group showed a positive correlation ( $r = 0.820$ ;  $P = 0.045$ ), but the eccentric-exercise group did not ( $r = 0.587$ ;  $P = 0.221$ ).

### Changes in fiber type composition

Fiber type composition in the two groups is shown in Fig. 4. In the eccentric-exercise group, the number of type IIb/d fibers was significantly lower than in the sham-operated group ( $P = 0.001$ ), whereas that of type IIa fibers was significantly larger than in the sham-operated group ( $P =$



**Fig. 5.** Relative  $\beta$ -connectin content [ $\beta/(\alpha+\beta)$ ] in the medial gastrocnemius muscle after a 20-day eccentric exercise. Values are means + SD.

0.017). There was no significant difference in the number of type I fibers between the two groups ( $P = 0.160$ ).

### Connectin isoform composition

A typical example of SDS-gel patterns and the content of  $\beta$ -connectin relative to total connectin in the two groups are shown in Fig. 5. The content of  $\beta$ -connectin isoform did not show significant difference between the groups.

## DISCUSSION

The present eccentric exercise protocol caused in the medial gastrocnemius muscle increases in the wet weight, cross-sectional area of type IIa fibers, and concomitant increase in isometric ankle flexion torque. The wet weight of trained medial gastrocnemius muscle (right) relative to the contralateral muscle (left) increased by ~11% in the eccentric-exercise group. Previous studies with rat medial gastrocnemius muscle have shown that the wet weight of the muscle increased by ~11% after a 20-day eccentric exercise (10 sessions) and by ~7% after a 28-day eccentric exercise (14 sessions) [23, 24]. The present result on muscular size is consistent with these previous studies and indicates that a repeated bout of moderate eccentric contractions effectively gives rise to muscular hypertrophy.

Muscular hypertrophy is generally associated with increases in contractile proteins and connective tissue proteins such as type I collagen [26], both of which may cause an increase in PRT concomitant to the increase in muscular strength. In particular, it has been shown that strenuous eccentric exercise causes an increase in connective-tissue content relative to the contractile element through damages of muscle fibers [27]. Therefore, it was initially predicted that repeated bouts of eccentric exercise would tighten the muscle and cause an increase in static PRT. Contrary to this prediction, the present eccentric exercise protocol caused a decrease in static PRT. On the other hand, the sham-operated group showed a positive correlation between static PRT and muscle weight, but the

eccentric-exercise group did not (Table 3). This suggests that we first confirm that the relationship found in the human study was also seen in untrained animals [28].

Several previous studies have shown that cytoskeletal protein connectin plays a significant role in determining the passive elastic properties of muscle fibers [14, 29]. Although measuring the absolute content of connectin within fibers is difficult due to its large molecular mass, its isoform composition ( $\alpha:\beta$ ) has been thought to be an indicator for the protein metabolism of connectin [20, 21, 30]. It has been shown that the relative content of  $\beta$ -connectin ( $\beta/\alpha+\beta$ ) is larger in fast type II fibers than in slow type I fibers [30], and the smaller  $\beta/\alpha+\beta$  may provide larger passive resistance to stretch. The present study showed that eccentric exercise training caused the transition from type IIb/d to type IIa fibers but no change in the number of type I fibers (Fig. 4). In addition, no significant change was seen in the connectin isoform composition after the period of training (Fig. 5). These results are consistent with the study by McGuigan et al. demonstrating a decrease in the number of type IIb/d fibers and no change in the connectin isoform composition in human muscles after an 8-wk explosive jump training [20] and suggests no direct relation between the observed reduction in static PRT and the muscle connectin isoform composition.

The joint stiffness may also be influenced by factors other than passive elastic properties of muscle fibers, e.g., connective tissues around the joint and muscle fibers, and proprioceptive muscular tone [31, 32]. In the present study, residual muscular tone after a stretch reflex might affect the value of static PRT, although the stress relaxation was allowed to proceed for a long period of time (90 s). Several studies with humans have shown that eccentric contractions cause changes in muscular tone and electromyographic activities [32–34]. Therefore, the present reduction in static PRT after eccentric exercise training may be related to any changes in muscle proprioceptive activities, e.g., an elevation of threshold for the stretch reflex.

On the other hand, a mechanical environment such as exercise training, unloading and immobilization has been shown to stimulate both synthesis [35] and breakdown [36] of type I collagen in connective tissues. In the present study, changes in protein metabolism might occur in type I collagen and other components, and the resulting restructure of connective tissue might cause a reduction of static PRT.

In conclusion, repeated bouts of moderate eccentric contractions caused in the rat medial gastrocnemius muscle increases in muscular size and strength, whereas the ankle-joint stiffness against dorsi-flexion was markedly reduced. The hypertrophied muscle showed a shift in fiber-type distribution from type IIb/d to type IIa and no change in connectin isoform composition, suggesting no substantial change in the passive elastic properties of mus-

cle fibers. The precise mechanism for the reduction of joint stiffness after a prolonged period of eccentric exercise needs further elucidation.

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