Role(s) of gravitational loading during developing period on the growth of rat soleus muscle fibers

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Adult male and female Wistar rats were mated and were given a commercial solid diet (CE-2, Nihon CLEA, Tokyo) and water ad libitum. The pups (~12 per litter) were kept with their mother until day 4. Then they were separated randomly into cage-control group and experimental groups (n = 8 per litter) and were maintained for 3 mo from postnatal day 4 to month 3, and the reloading was allowed for 3 mo in some rats. Single expression of type I myosin heavy chain (MHC) was observed in ~82% of fibers in 3-mo-old controls, but the fibers expressing multiple MHC isoforms were noted in the unloaded rats. Although 97% of fibers in 3-mo-old controls had a single neuromuscular junction at the central region of fiber, fibers with multiple nerve endplates were seen in the unloaded group. Faster contraction speed and lower maximal tension development, even after normalization with fiber size, were observed in the unloaded pure type I MHC fibers. These parameters generally returned to the age-matched control levels after reloading. It was suggested that antigravity-related tonic activity plays an important role in the gain of single neural innervation and of slow contractile properties and phenotype in soleus muscle fibers.

Tonic Activity during Posture Maintenance in a Gravitational Environment

The properties of neuromuscular junctions are also different between fast- and slow-twitch muscle fibers. Waerhaug and Lomo (31) reported that fast motoneurons form large ectopic junctions, whereas slow motoneurons form small ectopic junctions, concluding that the type of motoneuron, not muscle fiber, determines the fast or slow character of the neuromuscular fiber. Furthermore, the number of nerve terminal branching of extensor digitorum longus and soleus muscle fibers in the rats that performed a treadmill exercise for 30 days was significantly greater than those in the nonexercised controls (3), suggesting that the characteristics of neuromuscular junction are able to adapt to the activity levels. However, it is not known how gravitational unloading, which inhibits the growth-related fiber-type transformation (23), influences the characteristics of neuromuscular junction.

The present study was carried out to test the hypothesis that gravitational loading plays the essential role for the growth-related gain of slow-twitch properties in the soleus muscle fibers. Another main purpose of the study was to determine whether the muscular properties in the animals, which were hindlimb unloaded throughout the developing period, were reversible. Generally, the body weight of rats gradually increases until 12–14 wk after birth. Therefore, the hindlimb unloading by tail suspension was carried out for 3 mo from postnatal day 4 to inhibit the weight-supporting activities of hindlimb muscles. The responses to the same period of ambulation recovery were also studied to investigate the reversibility. Fiber phenotype, expression of MHC protein isoforms, fiber contractile properties, and number and size of neuromuscular junction in the soleus fibers were analyzed. The responses of muscle fiber characteristics to 3-mo reloading were also investigated.

MATERIALS AND METHODS

Animal Care and Hindlimb Suspension

All experimental procedures were conducted in accordance with the Japanese Physiological Society Guide for the Care and Use of Laboratory Animals and also followed the guiding principles of the American Physiological Society. The study was also approved by the Animal Use Committee at Osaka University and Japan Aerospace Exploration Agency.

Adult male and female Wistar rats were mated and were given a commercial solid diet (CE-2, Nihon CLEA, Tokyo) and water ad libitum. The pups (~12 per litter) were kept with their mother until postnatal day 4. Then they were separated randomly into cage-control and hindlimb-unloaded groups. The hindlimb unloading was performed as was reported elsewhere (23). Briefly, a narrow piece of

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adhesive tape was secured to the lower one-third of the tail. A second piece of tape was attached to the tape placed on the tail. In turn, this tape was connected to string tied to a horizontal bar at the top of the cage. The string was then manipulated to elevate the hindlimbs to avoid the contact with the floor and wall of the cage. The hindlimb-unloaded pups were returned to their mother for nursing for 1 h after every 5-h of unloading until postnatal day 21. Pups in the control group were also separated from their mother and followed the same feeding schedule. Water and both solid and powdered diets were supplied during the last week of the nursing period. All animals were housed in cages with wood shavings at all times. In addition, all pups were handled with rubber gloves at all times to avoid rejection by the dam.

After postnatal day 21, both groups of rats were separated from their mother, and the same amount of solid diet was fed. The amount of food supplied for each rat, which was completely eaten within ~12 h, was gradually increased from ~6 to ~20 g in accordance with growth. Hindlimb suspension was performed continuously. A sticky tape (~5 mm width and 3-cm length) with good cushion was placed longitudinally on the dorsal and ventral sides of the midtial of the hindlimb-unloaded rats. These tapes were further surrounded cross-sectionally by a tape. Such treatment was performed loosely to keep the blood flow intact. A string was inserted through the gap between the tail and tape and fastened to the roof of cage at a height allowing the forelimbs to support the weight, yet prevent the hindlimbs from touching the floor and the wall of the cage. The rats could reach the food and water freely by using their forelimbs. The attachment on the tail was changed every 2 wk so as not to inhibit the growth of tail and blood flow.

Hindlimb suspension was terminated at postnatal month 3. Each group of rats was further separated into four groups. Soleus muscles were sampled from one group immediately after 3 mo. The rats were anesthetized by intraperitoneal (ip) injection of pentobarbital sodium (5 mg/100 g body wt) during suspension to avoid any effects of acute loading. The remaining groups of rats were allowed an ambulation recovery in the cages. Tissue samplings were performed in the first, second, and third month. Temperature and humidity in the animal room with 12:12-h light-dark cycle were maintained at ~23°C and ~55%, respectively.

Recording of Electromyogram Activity

Activities of soleus muscles at rest on the floor and during suspension were estimated by recording electromyogram during the last week of unloading and 1-, 2-, and 3-mo ambulation recovery on the floor. Electrode implantation was performed following anesthesia with ip injection of pentobarbital sodium (5 mg/100 g body wt) during suspension to avoid any effects of acute loading. The remaining groups of rats were allowed an ambulation recovery in the cages. Tissue samplings were performed in the first, second, and third month. Temperature and humidity in the animal room with 12:12-h light-dark cycle were maintained at ~23°C and ~55%, respectively.

Preparation of Muscle for Analyses

Soleus muscles were removed bilaterally from rats anesthetized with ip injection of pentobarbital sodium (5 mg/100 g body wt) at postnatal day 4, month 3 [recovery (R) + 0-mo], 4 (R+1-mo), 5 (R+2-mo), and 6 (R+3-mo). The experiment was repeated until five male rats per group were obtained at each stage. Left soleus muscle was cleaned of excess fat and connective tissue and was weighed immediately. The muscle was then, carefully torn into longitudinal myofiber segments, including both the proximal and the distal tendon under the microscope, and the half segment was stored in the cellbanker (Nihon Zenyaku, Tokyo) at ~8°C until analyses. To measure the fiber contractile properties, another half segment was longitudinally tied to a glass bar by use of surgery thread and was placed in a cold 50% (vol/vol) glycerin solution (4°C) for 24 h. The glycerin solution was exchanged with freshly prepared glycerin solution, and the myofiber segments in the solution were kept at ~20°C until the analyses were performed. Right soleus muscle was pinned on a cork at an optimum length, frozen in liquid nitrogen-cooled isopentane, and stored at ~80°C until analyses. The midportion of the frozen muscle was then mounted perpendicularly on a cork by using optimum cutting temperature compound (Miles, Elkhart, IN) for immunohistochemical analyses. Because the muscle fibers from the 4-day-old rats were too short and the protein content was less, the analyses of contractile properties and gel electrophoresis for the single fibers were not performed. However, immunohistochemistry in the cross sections and electrophoresis in the whole muscle homogenates were performed to compare the growth between the 4-day- and 3-mo-old rats.

Distribution of Fibers with Various Phenotypes

Cross sections of the soleus muscle (10-μm thickness) in 4-day- and 3-mo-old groups were cut in a cryostat maintained at ~20°C. The immunohistochemical staining of MHC was performed in the cross sections by using a monoclonal antibody specific for fast (type II) or slow (type I) MHC isoform, i.e., primary antibody, NCL-MHCs and NCL-MHCf (Novocasta Laboratories), as described previously (23). Briefly, the avidin-biotin immunohistochemical procedure was used for the localization of primary antibody binding, according to the instructions for ABC kit (Vector Laboratories). Phosphate-buffered saline (PBS) was used as a buffer for the immunoglobulin G-class primary antibodies. The visualization for primary antibody binding site was performed with dianimonobenzidine tetrahydrochloride. The stained images were incorporated into a computer (Power Mac G3, Apple). The percent distribution of fibers expressing either I, II, or both I and II MHC were analyzed in the stained cross sections. At least 200 fibers were analyzed in each muscle.

Electrophoretic Analyses of MHC Isoform Expression

The expression patterns of MHC isoforms were determined in the single-muscle fibers, sampled from the right limb, in 3-, 4-, 5-, and 6-mo-old rats, as well as the whole muscle homogenates in 4-day-old pups. In 4-day-old samples, the insoluble (myofibrillar) proteins were

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extracted following the repeated homogenization in Tris-buffered saline containing 250 mM sucrose and 1% Triton X-100. The myofibrillar proteins were solubilized in the sodium dodecyl sulfate (SDS) buffer solution consisting of 0.1 M Tris·HCl, 5 mM ethylenediamine tetraacetic acid, 10% SDS, and 50 mM diithiothreitol.

The remaining portions (≈5-mm length) of right muscles in 3-mo-old groups, which were utilized for cross-sectional analyses, and whole muscle of 4-, 5-, and 6-mo-old groups were gradually thawed to room temperature in a low-calcium relaxing solution, as described previously (23). Single-fiber segments (at least n = 40 from each muscle) were mechanically isolated by using fine tweezers under a dissecting microscope. The individual fiber was solubilized in the SDS buffer solution.

Electrophoresis was carried out using Mini-Protein II dual-slab electrophoresis unit (Bio-Rad). To enhance the migration and separation of MHC isoforms, the separating gel containing 10% acrylamide and 30% glycerol was used. Gels were run at 80 V overnight in a refrigerator, maintained at 4°C, and were then stained with silver stain plus kit (Bio-Rad). In each gel, 10 μl of sample were loaded. Further, a “standard” lane was loaded with 10 μl of a mixture of homogenate of soleus and medial gastrocnemius muscles of adult rat and hindlimb muscles of neonatal rat to facilitate accurate determination of MHC isoform expression. The stained gels were computerized with the ImageScannerII (Amersham Biosciences). The amount of each MHC isoform expressed was quantified using the Scion image. The pattern of MHC expression was determined when the background of the gel was set at zero optical density, because the amount of protein loaded in each lane was not necessarily constant due to the different fiber size.

Fiber Contractile Properties

On the day of the experiment, single-muscle fibers, sampled from the left limb, were isolated in relaxing solution (35). The relaxing solution consisted of 10 mM EGTA, 3.5 mM MgATP, 1.5 mM MgCl2, and 20 mM PIPES (pH 7.0, 20°C). The ionic strength was adjusted to 0.2 M with potassium methansulfonate. A single fiber was transferred to an experimental chamber (0.3 ml volume) filled with the relaxing solution containing 250 mM sucrose and 1% Triton X-100. The myofiber segments sampled from the left limb, stored in the cellbanker, were instantly thawed at 35°C. Collagens were digested in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 0.2% type I collagenase, 1% antibiotics, and 10% newborn calf serum for 4 h at 35°C. Then the segments were fixed with 4% buffered formaldehyde for 10 min and rinsed with PBS. Subsequently, whole single fibers were isolated from tendon-to-tendon using fine needles and carefully collected by using pipette to avoid scratching of the fibers (34). Fibers collected from a muscle were immersed in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum. Working solution of collagenase was gel purified to remove the cistrain, which supposedly strips the basal lamina of the fiber (4).

The collected single fibers were incubated with α-bungarotoxin conjugated to fluorescein (Molecular Probes) diluted 1:100 with PBS to visualize AchR α-subunits at neuromuscular junction. After staining, the single fibers were rinsed with PBS and stored in PBS at 4°C until analysis. Immediately before the analysis, the fibers were mounted on a slide glass using 50% glycerol and coverslips with “struts” of hardened nail polish on the corners to minimize fiber compression.

Confocal Microscopy

A Fluoview confocal microscope with an argon laser (488 nm of peak wavelength) (Olympus, Tokyo) was used to analyze the number and diameter of AchR clusters. The number of AchR clusters per fiber was counted in α-bungarotoxin-labeled fibers. The AchR cluster was also scanned using the proper filter sets for fluorescein, and the mean diameter of AchR cluster was measured. The levels of AchR cluster diameter were normalized at 2.5-μm sarcomere length. A maximum-intensity projection rotated orthogonally to the long axis of the fiber was produced from the stack, and the fiber CSA was measured at the portion where the AchR cluster diameter was measured. Then the
diameter was normalized by the observed fiber CSA. The fibers with damaged regions and adipocytes were omitted from the analyses.

Statistical Analysis

Values are expressed as means ± SE. Statistical significance was examined by two-way analysis of variance, followed by Scheffé's post hoc test. Differences were considered significant at the 0.05 level of confidence.

RESULTS

Properties of Muscle Fibers

MHC phenotype. SINGLE FIBERS. According to the analyses of single muscle fibers, the percent distribution of fibers expressing pure type I MHC was 82% in the control rats, but was only 53% in the unloaded rats at the age of 3 mo (Fig. 1, P < 0.05). The percentage of pure type I MHC fibers significantly increased within 1 mo after recovery (+30%, P < 0.05), but was still insignificantly (7%) less than the age-matched control level, even after 3 mo. The distribution of type I+IIa MHC fibers of unloaded muscle tended to be greater than that of controls (P > 0.05) at the end of suspension. Although the distribution of these fibers in the control tended to decrease thereafter, that in the unloaded group remained high, at least up to month 5 (P < 0.05). The number of type IIa MHC fibers after 3-mo unloading was identical to that in control. These fibers in the control group decreased during recovery, and they were not detected after 3 mo. The reduction of these fibers in the unloaded group was more rapid. They were not detected even after 1 mo. A de novo appearance of fibers with multiple expression of MHCs, e.g., I+IIa+IIx, I+IIa+IIx+IIb, I+IIa+IIb, I+IIb, or IIa+IIx, and with pure IIx was noted in the unloaded muscles at the end of suspension. However, these fibers were decreased during the recovery period. The fibers expressing I+IIa+IIx+IIb, I+IIa+IIb, I+IIb, IIa+IIx, and IIx MHC were not seen 1 mo after the termination of suspension.

WHOLE MUSCLE. The MHC expression of single fibers at the age of 4 days was not analyzed, because the protein content was not enough. However, it was determined in whole muscle. The percent distribution of fibers expressing pure type I or II MHC, or coexpressing both type I and II MHC, analyzed by immunohistochemistry in the cross section of muscle is shown in Fig. 2. The percentages of fibers expressing type I, I+II, and II MHC were 55, 4, and 41%, respectively, in soleus muscle of newborn rats at the 4th day after birth. Significant shift toward slow-twitch type was noted following 3-mo growth in a 1-G environment. The percent distribution of type I and I+II fibers was increased to 79 and 16%, respectively, and that of type II fibers was decreased to 5%, on the contrary (P < 0.05). However, such a growth-associated shift toward slow-twitch type was inhibited by unloading. The percent type I fiber of 3-mo-old unloaded rats was similar to that of 4-day-old preunloading level. Also, growth-associated decrease of percent type II MHC fiber was inhibited, although the mean level was significantly decreased by 19% (P < 0.05). The increase of fibers coexpressing type I and II MHC was further promoted, as well.

Neuromuscular junction. The neuromuscular junctions were identified by labeling the AchR using α-bungarotoxin (Fig. 3A). The mean diameter of endplate in the control group was increased during 3 mo (3.8 times vs. day 4 after birth, P < 0.05, Fig. 3B). The growth-related increase of its size was insignificantly inhibited by unloading (P > 0.05). The difference of the endplate diameter between the control and unloaded groups became significant statistically after 2 and 3 mo of recovery (P < 0.05). The endplate diameter per fiber CSA in the control group was slightly, but significantly, elevated during the 3-mo postnatal development (29%, P < 0.05, Fig. 3C). This increase in the unloaded group was prominent (265% vs. the presuspension, and 182% vs. the age-matched control, P < 0.05). However, its level was gradually decreased toward the control level and was normalized 2 mo after recovery.

All of the fibers, analyzed at the 4th day after birth, had one nerve endplate at the central region (Fig. 4A). The percent distributions of the fibers containing a single endplate at the postnatal month 3 were 97.2 and 59.5% in the control and unloaded group, respectively (P < 0.05). Multiple endplates were noted in many fibers of the unloaded group. Approximately 32, 7, and 1% of the fibers had two, three, and even five endplates at the 3rd and 4th month after birth, respectively (P < 0.05). The number of endplates in the unloaded group was further increased after unloading (P < 0.05).

Fig. 1. The distribution of fibers expressing various types of myosin heavy chains (MHCs), analyzed by gel electrophoresis in single-muscle fibers. The typical patterns of MHC isoforms in the myofibrillar extract (4 day) and muscle fibers (3 mo) are also shown. Values are means ± SE. Significantly different from the level immediately after †3-mo unloading or cage housing and §age-matched control: P < 0.05.
endplates, respectively. On the contrary, only ~3% of control fibers possessed two endplates. Fibers with more than two endplates were not seen in the control group. The fibers with the multiple endplates were, however, decreased, and those with a single endplate were increased within 1 mo during reloading on the floor. Multiple endplates were not observed in the control muscle fibers at the age of 4 mo, but the fibers containing two endplates were still observed in the unloaded group, even during the 3-mo recovery (Fig. 4, B–D).

Contractile properties. The contractile properties of the skinned muscle fibers, followed by the analyses of fiber phenotype using gel electrophoresis, were measured. However, the results from fibers expressing only pure type I MHC were used as the data, because the distribution of other types of fibers was very low, and these fibers were not always detected in some samples. The typical patterns of tension development in the control and unloaded group were shown in Fig. 5A. Changes in the CSA of fibers used for the analysis of contractile properties were also shown (Fig. 5B). The levels of absolute (Fig. 5C) and CSA-normalized isometric tension (Fig. 5D) in the soleus muscle fibers were 97 and 81% less at the end of 3-mo unloading than for the age-matched control rats, respectively (P < 0.05). The maximum tension development gradually increased toward the control levels in response to reloading, although the mean levels were still less than the age-matched controls after 3 mo, respectively (P > 0.05). The V0 was 97% greater at the end of 3-mo unloading than for the age-matched control rats (P < 0.05), but was gradually decreased toward the control level following recovery (Fig. 5E). The sensitivity of fibers to Ca2+ was evaluated as pCa50, which is the Ca2+ concentration when one-half of the maximum tension was obtained (Fig. 5F). The mean pCa50 was 5.44 and 5.84 in the unloaded and control fibers 3 mo after birth, respectively. The pCa50 level in the control fibers was constant throughout the experimental period, but that in the unloaded group gradually increased to 5.61, 5.70, and 5.79 after 1, 2, and 3 mo of reloading, respectively, although it was not fully normalized to the control level (P > 0.05).

Activity pattern of muscle in vivo. Tonic activity was noted in the soleus muscle of cage control rats at rest on the floor (Fig. 5G). The muscle activity estimated by the recording of electromyogram was significantly inhibited by unloading, as was reported elsewhere (17). Also, the typical activity pattern during the last week of 3-mo unloading was phasic. However, the tonic activity pattern was recovered after 1-mo ambulation in the cage.

DISCUSSION

Effects of gravitational loading or unloading on the gain of the characteristics in soleus muscle fibers were studied in rats. The tail suspension from the postnatal day 4 to month 3 inhibited the growth-associated transformation of fibers, such as MHC expression, decrease of the number of neuromuscular junction, and/or contractile properties, toward slow-twitch type. However, these parameters generally returned to the age-matched control levels after 3-mo reloading.

Body and Muscle Weight

We previously reported that the growth-related gain of both body weight and the absolute soleus muscle weight was remarkably inhibited, if the hindlimb suspension was performed from the postnatal day 4 to month 3 (14). The increase in the weight of the soleus muscle relative to the body weight was also inhibited by the prolonged unloading. The growth-associated increase of fiber CSA was also inhibited by 3-mo unloading (~69% vs. the age-matched control). Such unloading-related inhibition of muscle growth was associated with the inhibited increase of satellite cells and myonuclei. Furthermore, the regrowth of the fibers was observed, if the unloading was terminated and the reloading was allowed for 3 mo. Therefore, it was suggested that the satellite cell-related stimulation in response to mechanical load and/or neural activity played an essential role in the cross-sectional growth of soleus muscle fibers.

Muscle Activity

The integrated electromyogram activity of soleus muscle during the last week of 3-mo suspension, which was analyzed in the same experiment as the present study, was ~86% less than that in the age-matched control rat at rest on the floor (19). Chronic inhibition of soleus muscle activity was also observed during 9-wk suspension of adult rats (20). These results clearly indicated that neural activity of muscle was inhibited during suspension. Furthermore, the result illustrated in Fig. 5G showed that the activity of soleus muscle was changed to phasic pattern during the chronic unloading. Dorsiflexion of ankle joints in rats, hindlimb suspended for 3 mo, was inhibited due to the morphological changes in hindlimb bones (19, 20). Therefore, the degree of passive stretching of soleus muscle at a quadrupedal prone position on the floor was less than that in
normal controls. However, the tonic activity, noted in the control rats, was recovered following 1-mo ambulation after 3-mo suspension.

**Fiber Phenotype**

Although rat soleus muscle at the age of 4 days was composed of ~55% of type I MHC fibers, ~4% of type I+II MHC fibers, and ~41% of type II MHC fibers (Fig. 2), a fiber-type transformation, such as MHC II→I+II→I, was induced during the 3-wk postnatal development on the floor (23). Fiber transformation toward slow-twitch type was further progressed during 3 mo of growth in the control rats, and the percent distributions of type I, I+II, and II MHC fibers became ~79, ~16, and ~5%, respectively. However, the increase in the distribution of pure type I MHC fibers was completely inhibited in response to unloading for 3 mo (55%). The fibers coexpressing type I and II MHC, on the contrary, increased in unloaded soleus muscle (26%), indicating that the growth-associated shift of fiber phenotype toward slow type was suppressed during hindlimb unloading. We also reported previously that growth-associated shift of fiber phenotype was completely suppressed by hindlimb unloading during the lac-

Fig. 3. **A**: pictures showing the location of nerve endplates (shown by arrowhead) in single muscle fiber of 3-mo-old rats. **B**: endplate diameter. **C**: the ratio of endplate diameter to fiber cross-sectional area (CSA). Values are means ± SE. Significantly different from *4-day-old control, †immediately after 3-mo suspension or cage housing, and §age-matched control: P < 0.05.
The soleus muscle of the neonatal rats with the age of 7 days, of embryonic and neonatal isoform of MHC was observed in with metabolic activity. It was also reported that the expression and/or neural activities of muscle are also generally associated but the precise mechanism is still unclear, since the mechanical notype is predominantly dependent on the postnatal activity, data suggest that the growth-related change in the fiber phe-
tation period and the mean distributions of type I, I+II, and II MHC fibers in the unloaded rat soleus were still ~55, ~2, and ~43% at the age of 21 days, respectively (23).

The fiber phenotype was recovered toward the control level in response to ambulation, although the percentage of pure type I MHC fibers was still significantly (7%) less than that of the age-matched controls, even after 3 mo. Furthermore, chronic exposure of rats to 2-G environment between postnatal day 4 and month 3 caused a faster transformation of soleus fibers toward slow-twitch type (unpublished observations). These data suggest that the growth-related change in the fiber phenotype is predominantly dependent on the postnatal activity, but the precise mechanism is unclear, since the mechanical and/or neural activities of muscle are also generally associated with metabolic activity. It was also reported that the expression of embryonic and neonatal isoform of MHC was observed in the soleus muscle of the neonatal rats with the age of 7 days, but the expression levels decreased during the first 3 wk after birth (1, 2). However, no bands for the embryonic and neonatal isoforms were visualized by a silver staining in the soleus muscle of 4-day old rats in the present study. Although the precise mechanisms are still unclear, it was indicated that the modulation of the fiber phenotype in the soleus muscle was postnatally controlled.

Gravitational unloading by exposure to space environment or by simulation models, such as hindlimb suspension (17, 34) or bed rest (25), causes the shift of soleus fibers toward fast-twitch type. It was reported that these phenomena were associated with the decreased turnover rate of ATP (22), as well as the contractile activity-dependent decrease of intracellular calcium concentration (6). On the other hand, the shift of fiber phenotype toward slow characteristics is usually associated with increased levels of the calcineurin signaling, followed by the contractile activity of muscles (6). However, we reported a metabolic modulation of fiber phenotype, which is unrelated to the mechanical stimuli (18). The percentage of slow fibers in soleus and extensor digitorum longus muscles of rats was increased in response to lowered high-energy phosphate levels due to depletion of creatine caused by feeding creatine analog β-guanidinopropionic acid. The daily voluntary activity and electromyogram activities of soleus in these rats were even lower than for controls. Shift of muscle toward slow-twitch type with higher levels of mitochondrial enzymes was also observed following the chronic depletion of high-energy phosphates by exposure of frogs to cold (21). Taken together, it is suggested that the regulation of fiber phenotype in response to the altered level of antigravity activity may be modulated by metabolic stimuli, which is influenced by me-
chano- and neural activity.

**Fiber Contractile Properties**

The larger fibers generally develop greater force than small fibers, because of the greater number of cross bridges. Thus the absolute tension production was ~97% less in the unloaded muscle fibers with smaller CSA in the present study (Fig. 5C). However, the relative tension normalized by fiber CSA was also less in the unloaded than control fibers (Fig. 5D). It has also been reported that the reduction of relative tension production per CSA was seen in the atrophied human soleus muscle fibers following 4-mo bed rest (35). The relative tension per CSA after 2 and 4 mo was 27 and 42% less than the pre-bed-rest level, respectively. Such phenomena may be related to a greater reduction of contractile proteins than other structural or metabolic proteins following unloading. But the precise mechanism is still unknown.

It was also noted that the $V_o$ of fibers expressing pure type I MHC in the unloaded group was ~97% greater compared with that of the aged-matched control (Fig. 5E). The expression of MHC has been known as a major factor that determines the shortening velocity, because myosin ATPase activity is dependent on the MHC phenotypes. However, the MHC transformation may not be the sole cause for the changes in $V_o$, because the unloading-related increase of $V_o$ was observed even in pure type I MHC fibers.

The expansion of the lattice spacing has been reported as one of the mechanisms responsible for increased $V_o$ in type I MHC fiber (27). The lowered $Ca^{2+}$ sensitivity was also observed in
Fig. 5. Contractile properties of single fibers of soleus muscle. A: pictures showing the typical patterns of tension development in single fiber of control and unloaded rats immediately after 3-mo cage housing or unloading. B: changes in the CSA of fibers, which were used for the analysis of contractile properties. C: absolute maximally activated isometric tension. D: relative maximally activated isometric tension per fiber CSA. E: unloaded shortening velocity. FL, fiber length. F: sensitivity of the fibers to calcium: calcium concentration when one-half of the maximum tension was obtained. G: typical patterns of electromyogram in soleus muscles of rats at rest on the floor and during hindlimb suspension, recorded during the last week of 3-mo unloading period. Recovery of tonic electromyogram activity was noted in the unloaded rats following 1-mo ambulation. Values are means ± SE. Significantly different from the level immediately after †3-mo unloading or cage housing (R+0-mo) and §age-matched control: *P < 0.05.
the unloaded muscle fibers (Fig. 5F). It is generally accepted that the Ca\(^{2+}\) sensitivity in the muscle fibers is dependent on the isoform of troponin C (26, 29) and/or the cooperativity between thin and thick filaments (15). It has been reported that the intracellular free Ca\(^{2+}\) concentration in soleus muscle with unloading-induced atrophy was elevated in a resting state (11). Unloading might cause the alterations of intracellular Ca\(^{2+}\) status in muscle fibers, resulting in modification of the Ca\(^{2+}\) sensitivity of myofiber. reloading promoted the normalization of the fiber characteristics.

Neuromuscular Junctions

Although the muscle fibers are innervated polineuronally at birth, the number of neuromuscular junction is decreased during the postnatal development, and the reduction is completed by the second week after birth (5). At postnatal day 4, a single group of junction area was noted at the middle portion in all of the fibers analyzed, suggesting that the aggregation of AchR was already formed normally (Fig. 4A). Approximately 97% of muscle fibers of control rats had a single junction area at the age of 3 mo, and ~3% of fibers had 2 junctions. However, the fibers containing one junction area was only ~60% in the unloaded fibers, and multiple junction areas were noted in ~40% of fibers. The single junction in the fiber was located at the middle region of the fiber length. Double junctions were located at the proximal and distal regions of the fibers.

The total fiber number of the soleus muscle at the 4th day after birth was ~800 and was increased to ~2,500 following the normal 3-mo growth (14). The growth-related fiber formation was not influenced, even if the rats were hindlimb unloaded between postnatal day 4 and month 3. It is suggested that the unloading may cause an abnormal innervation in some newly formed fibers during the postnatal development, since all fibers in 4-day-old rats had single innervation. However, the number of junctions was normalized within 1-mo reloading. It is indicated that the formation and/or location of the junction area in soleus muscle fibers may be regulated, in part, by the gravity-dependent motor activity after birth.

The fibers coexpressing the multiple isoforms of MHCs were noted in the soleus muscle fibers at the end of 3-mo unloading. However, these fibers disappeared within 1 mo of reloading. It is speculated that the appearance of fibers coexpressing various types of MHC may be closely related to the unloading-associated abnormal activity due to the plural inputs from the multiple junctions. The relationship between the location of junctions and phenotype or function of fiber is unclear, since the matured muscle fibers are innervated by a single motoneuron generally, regardless of their phenotype (5). However, the multiple innervation may affect the motor performance. The abnormal patterns of electromyogram in hindlimb muscles, such as coactivation of dorsiflexor and plantarflexor muscles, were observed during walking on the floor after chronic unloading (20). Walton et al. (32) also reported that newborn rats that were hindlimb unloaded from postnatal days 8–13, could not swim. These findings suggest that the gravitational unloading during the critical period for development of the motor system causes a failure of morphological and functional growth of skeletal muscle fibers.

Waerhaug and Lomo (31) reported that fast and slow motoneurons form large and small ectopic junctions, respectively, although another report indicated that morphological properties of both nerve terminal and endplate were similar between MHC I and IIa fibers (27). The nerve terminals in fibers of extensor digitorum longus and soleus muscle of rats that performed 1-h running training at 30 m/min for 30 days had significantly greater number of branching than those in the nonexercised controls, although the perimeters were identical (3). Deschenes et al. (7) reported that endplate areas of soleus muscle fibers, associated with an atrophy and shift toward fast-twitch type, were at least 30% greater in rats after 16 days of spaceflight than the ground-based controls. These reports suggest that the size of neuromuscular junction is able to adapt to the motor activity patterns. In the present study, the relative size was prominently greater in the unloaded fibers, if the diameter of junction was normalized by fiber CSA (Fig. 3C). This phenomenon, which is generally noted in fast-twitch fibers (31), may be related to the phasic activity of the soleus muscle during suspension (Ref. 28 and Fig. 5G). However, the size was normalized when the activity pattern of the muscle became tonic in response to reloading on the floor, suggesting that the small junction in the soleus muscle fibers, seen in rats grown on the floor normally, may be closely related to the postnatal tonic activity. These results indicate that the growth of the size of neuromuscular junction is also dependent on the gravitational load or the pattern of activity. Waerhaug and Lomo (31) reported that the type of motoneuron determines the fast or slow character of the neuromuscular junction. However, the data in the present study suggest a significant contribution of the activity of muscle fiber itself.

Conclusion

The role(s) of gravitational loading on the growth of rat soleus muscle fibers was studied. The number of slow-twitch fibers and/or type I MHC expression was increased during the 3-mo growth in 1-G environment. If the soleus muscle were unloaded by tail suspension during the developing period, such fiber transformation was inhibited, and fibers expressing multiple MHC isoforms were noted. Approximately 97% of fibers of control rats contained only one neuromuscular junction at the central portion, but multiple endplates were observed at various portions of fiber in the hindlimb-unloaded group. Furthermore, the hindlimb unloading inhibited the growth-related increase of the maximal tension development, even in the fibers expressing type I MHC alone. Shortening velocity of these fibers was ~97% faster than that in the age-matched controls. These contractile properties in the unloaded group were related to inhibition of electromyogram activity and shift from tonic, which was noted in cage controls, to phasic patterns. However, unloading-related inhibition of normal growth of fibers was generally normalized to the control levels after reloading. It was suggested that the mechanical loading and tonic activity may be essential in the gain of single neural innervation and of slow contractile properties and phenotype in soleus muscle fibers.

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GROWTH-RELATED FIBER PHENOTYPE TRANSFORMATION

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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