Essential role of satellite cells in the growth of rat soleus muscle fibers

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Kawano F, Takeno Y, Nakai N, Higo Y, Terada M, Ohira T, Nonaka I, Ohira Y. Essential role of satellite cells in the growth of rat soleus muscle fibers. Am J Physiol Cell Physiol 295: C458–C467, 2008.—Effects of gravitational loading or unloading on the growth-associated increase in the cross-sectional area and length of fibers, as well as the total fiber number, in soleus muscle were studied in rats. Furthermore, the roles of satellite cells and myonuclei in growth of these properties were also investigated. The hindlimb unloading by tail suspension was performed in newborn rats from postnatal day 4 to month 3 with or without 3-mo reloading. The morphological properties were measured in whole muscle and/or single fibers sampled from tendon to tendon. Growth-associated increases of soleus weight and fiber cross-sectional area in the unloaded group were ~68% and 69% less than the age-matched controls. However, the increases of number and length of fibers were not influenced by unloading. Growth-rate increases of the number of quiescent satellite cells and myonuclei were inhibited by unloading. And the growth-related decrease of mitotically active satellite cells, seen even in controls (20%, P > 0.05), was also stimulated (80%). The increase of myonuclei during 3-mo unloading was only 40 times vs. 92 times in controls. Inhibited increase of myonuclear number was not related to apoptosis. The size of myonuclear domain in the unloaded group was less and that of single nuclei, which was decreased by growth, was larger than controls. However, all of these parameters, inhibited by unloading, were increased toward the control levels generally by reloading. It is suggested that the satellite cell-related stimulation in response to gravitational loading plays an essential role in the cross-sectional growth of soleus muscle fibers.

IT IS WELL REPORTED THAT GRAVITATIONAL unloading by actual spaceflight and/or hindlimb unloading causes atrophy in antigravity muscle, soleus, of matured rats (1, 2, 18, 26, 28, 30–33, 39). Loss of myonuclei is associated with the atrophy of soleus muscle fibers (1, 2, 26). On the contrary, muscle fiber size and the number of myonuclei in the soleus muscle of rats rapidly increase during the early development (26, 31). However, the growth-related increases of fiber size and myonuclear number are not induced if the gravitational loading is inhibited by tail suspension between postnatal day 4 and 21 (31). Furthermore, McCall et al. (23) reported that functional overload-associated fiber hypertrophy and increase of myonuclear number were promoted in soleus muscle fibers of adult rats if the overload was combined with the administration of growth hormone and insulin-like growth factor 1. These results clearly indicated the importance of myonuclei as genetic machinery for regulation of the skeletal muscle mass. However, it is still unclear whether the growth-related accretion of myonuclei is genetically programmed or postnatal activity dependent.

Muscle satellite cells are myonuclear precursors lying between the sarcolemma and the basal lamina of myofiber (22), and the myonuclear accretion occurs through the incorporation of satellite cell nuclei into the growing myofibers (24). Quiescent satellite cells are adhered to the myofiber with M-cadherin (17), and proliferation and differentiation are controlled by several growth factor families (11, 37) or nitric oxide (3). It is also reported that satellite cells are activated when the muscle is overloaded (9) or injured (35). However, the role of satellite cells in the growth and development of muscle fibers is still unclear.

Therefore, roles of gravitational loading on the differentiation and development of soleus muscle fibers of rats were studied in the present study to test the hypothesis that muscle fiber formation and growth during the developing period are load dependent. To examine the hypothesis, the effects of long-term unloading during the developing period and/or of reloading after the termination of unloading were investigated. Particular attention was paid to investigate the role of satellite cells in the development of antigravity muscle fibers.

MATERIALS AND METHODS

Animal Care and Hindlimb Unloading

All experimental procedures were conducted in accordance with the guidelines outlined by the Japanese Physiological Society and American Physiological Society and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). 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, Japan) 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 (31). Briefly, a narrow piece of adhesive tape was secured to the lower 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 a string tied to a horizontal bar at the top of the cage. The string was then manipulated to elevate the hindlimbs to avoid contact with the floor and walls of the cage.

Continuous hindlimb unloading during the lactation period made it difficult to maintain normal nursing. Therefore, the hindlimb-unloaded pups were returned to their mother for nursing for 1 h after every 5 h of unloading until postnatal day 21, as was reported previously (31). Pups in the control group were also separated from their mother at day 4.
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 unloading 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 midtail 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 the 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 to not inhibit the growth of tail and blood flow.

Hindlimb unloading 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 with intraperitoneal (ip) injection of pentobarbital sodium during suspension to avoid any effects of acute loading. The remaining groups of rats were placed into reloaded groups and allowed accommodation in the cage. Tissue samplings were performed after 1, 2, and 3 mo. 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

Both control and hindlimb-unloaded rats were killed at postnatal day 4, month 3 (recovery period (R) + 0 mo), month 4 (R + 1 mo), month 5 (R + 2 mo), and month 6 (R + 3 mo). The experiment, mentioned above, was repeated until five male rats per group were obtained at each stage. The rats were given a single ip injection of thymidine analog 5-bromo-2′-deoxyuridine (BrDU, 100 mg/kg body wt) (Sigma-Aldrich, St. Louis, MO) ~48 h before the samplings. The rats were anesthetized with ip injection of pentobarbital sodium (5 mg/100 g body wt), and soleus muscles were removed bilaterally.

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, Japan) at ~80°C until analyzed. Right soleus muscle was pinned on a cork at an optimum length, frozen in liquid nitrogen-cooled isopentane, and stored at ~80°C until analyzed. Midportion of the frozen muscle was then mounted perpendicularly on a cork by using optimum cutting temperature compound (OCT, Miles, Elkhart, IN) for cross-sectional immunohistochemical analyses.

Muscle Fiber Number

Cross section of the soleus muscle (10-μm thickness; n = 5 in each group at each stage) was cut in a cryostat maintained at ~20°C and was stained using a monoclonal antibody specific for fast (type II) myosin heavy chain isoform (Novocastra Laboratories), as described previously (31). 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 dianinobenidine tetrahydrochloride. The stained image was incorporated into a computer (Power Mac G3, Apple). The number of total fibers was counted in the stained whole cross sections at the midportion of muscle.

Immunohistochemistry and Nuclear Labeling

The myofiber segments stored in the cellbanker were instantly thawed at 35°C. Collagens were digested in Dulbecco’s modified Eagle’s medium (DMEM, 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 with fine needles and were carefully collected with pipettes to avoid scratching of the fibers (39). Fibers collected from a muscle were separated into two tubes (tube 1 and 2) and immersed in DMEM containing 10% newborn calf serum. Working solution of collagenase was gel purified to remove clostripain, which may strip the basal lamina of the fiber (5).

Immunohistochemical counterstaining of BrdU and M-cadherin was performed in the single fibers, as described previously (25, 26, 39). The single fibers in tube 1 and 2 were kept in 1% Triton X-100 diluted with PBS for 15 min. Subsequently, the single fibers in tube 1 and 2 were blocked with 10% normal goat serum diluted with PBS for 15 min and incubated overnight with a primary monoclonal antibody specific to BrdU (Becton Dickinson) and polyclonal antibody specific to M-cadherin (Santa Cruz Biotechnology) diluted 1:20 with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin (BSA), respectively. The M-cadherin-positive (quiets satellite cells) and BrdU-positive nuclei (mitotically active satellite cells) were visualized with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch) diluted 1:50 with PBS containing 0.5% Tween 20 and 0.5% BSA. The antibody-reacted fibers in tube 1 and 2 were further counterstained for the fiber nuclei (myonuclei + satellite cells) with propidium iodide (PI) (25 μg/ml PBS) for 10 min. After staining, the single fibers were rinsed with PBS and stored in PBS at 4°C until analyzed. Immediately before the analysis, the fibers were mounted on a slide glass in 50% glycerol with coverslips with “struts” of hardened nail polish on the corners to minimize fiber compression.

Labeling of Apoptotic Myonuclei

The remaining segments in the right soleus muscles of control and unloaded rats in the R + 0-mo group were gradually thawed to room temperature in a low-calcium relaxing solution, as described previously (31). Single fiber segments (n = 20 from each muscle) were mechanically isolated by using fine tweezers under a dissection microscope, placed on gelatin-coated glass slide, air dried, and stored at ~5°C until analyzed. Mechanical isolation of single fibers has been shown to strip off the basal lamina of muscle fiber including satellite cells (31). The apoptotic myonuclei were labeled using the terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) method as described previously (1). Briefly, the fibers were fixed with 4% buffered formaldehyde for 1 h and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 5 min. Subsequently, the fibers were incubated in the reaction buffer containing terminal deoxynucleotidyl transferase (Roche Diagnostic) for 1.5 h at 37°C. After the reaction, the fibers were rinsed with PBS and mounted in the mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI). The DAPI-labeled total myonuclei and TUNEL-positive apoptotic myonuclei were counted under a fluorescent microscope. The percent distribution of TUNEL-positive myonuclei per observed myonuclei (~1,000) was calculated in each fiber.

Confocal Microscopy

A Fluoview confocal microscope with an argon laser (488 nm of peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and peak wavelength) and a He-Ne laser (543 nm of peak wavelength, Olympus, Tokyo) was used for the analyses of the length and
The numbers of the nuclei, double-labeled by PI and FITC, per fiber were counted in BrdU and M-cadherin-labeled fibers to measure the quiescent and mitotically active satellite cells. The total number of nuclei per fiber was also counted in the same fiber. A maximum-intensity projection rotated orthogonally to the long axis of the fiber was then produced from the stack, and the fiber CSA was measured at 3 nonoverlapping regions randomly chosen along the fiber length. Myonuclear domain (7, 14), which is the cytoplasmic volume per myonucleus, was calculated by multiplying the fiber CSA and length and dividing by myonuclear (except the satellite cell) number per fiber. A series of the repeated scans using the proper filter sets for PI was taken at each 0.1 μm of orthogonal step through the entire Z thickness of a middle portion of the long axis of fiber. The three-dimensional myonuclear volume was then measured using 3D Viewer (Ratoc System Engineering). Finally, the mean emission intensity of PI was measured in each myonucleus to determine the content of myonuclear DNA. The emission intensity of fluorescence was quantified as 4,096 levels of scale. The fiber length and the length of 10 consecutive sarcomeres, randomly chosen from three nonoverlapping regions along the fiber length, were also measured in each fiber by Nomarski optic scan using calibrated measurement software (Olympus).

### Statistical Analysis

Values are expressed as means ± SE. Statistical significance was examined by two-way ANOVA, followed by Scheffé’s post hoc test using StatView packaging software (HULINKS, Tokyo, Japan). Differences obtained by Scheffé’s post hoc test were considered significant at the 0.05 level of confidence.

### RESULTS

#### Body and Soleus Weights

In the control rats, body weight and the absolute soleus wet weight were increased 34 and 65 times, respectively, during the 3 mo of development (vs. 4-day-old pups; *P* < 0.05; Table 1).

![Fig. 1. A: cross sections of the midbelly region of soleus, which were immunohistochromically stained by using a monoclonal antibody specific to type II myosin heavy chain. B: total fiber number of soleus muscle of 4-day-old and 3-mo-old rats. C: fiber cross-sectional area. D: fiber length and total sarcomere number in whole single fiber. Values are means ± SE; *n* = 5 for each group. *,$,,$Significantly different from the levels at day 4, immediately after 3-mo unloading or cage housing (3-mo), and the age-matched control, respectively: *P* < 0.05; Table 1.](https://www.ajpcell.org/content/295/5/C460)
However, the growth-related increases of these weights in the unloaded rats were only ~20 and 21 times, respectively ($P < 0.05$). These levels in the unloaded rats were ~42% and 68% less than those of the age-matched controls ($P < 0.05$). Although the weight of soleus relative to body weight in the control rats increased from 18.3% at postnatal day 4 to 40.6% at 3 mo, that in the unloaded rats remained unchanged (20.7%). The body weight and the absolute and relative soleus weight in the unloaded rats increased during the ambulation recovery but were still less than those of the age-matched controls even after 3 mo ($P < 0.05$).

**Properties of Muscle Fibers**

*Morphology.* Figure 1A shows the cross sections of the midbelly regions of soleus muscles in control and unloaded rats at the age of 3 mo. The mean muscle CSA of the unloaded group was approximately one-fifth of the age-matched control ($P < 0.05$). However, the growth-associated increase of total fiber numbers was not inhibited by unloading (Fig. 1B). The total number of muscle fibers was equally increased from ~800 at postnatal day 4 to ~2,500 at postnatal month 3 in both control and unloaded rats ($P < 0.05$).

The mean CSAs of fibers, sampled from tendon to tendon, in both groups were increased during 3 mo (Fig. 1C, $P < 0.05$). However, the growth was significantly inhibited by unloading. The mean CSA at the end of 3-mo unloading was only 31% of the age-matched control ($P < 0.05$). Although the fiber size was gradually increased in response to ambulation recovery, complete normalization to the control level was not reached even after 3 mo ($P < 0.05$). The mean level of control group was stable during the 3-mo recovery period. The longitudinal growth of muscle fibers was not influenced by unloading (Fig. 1D). The fiber length and the total sarcomere number were the same in both groups throughout the experimental period.

**Myonuclei.** Significant effects of unloading were noted in the distribution and morphological properties of myonuclei (Figs. 2 and 3). Myonuclear numbers per whole muscle fiber...
were still only
They were also increased even during the 3-mo unloading but were still only ~40% and ~45% of the age-matched controls, respectively (P < 0.05). The number of myonuclei in the unloaded group was gradually increased in response to ambulation recovery but was still less than that in the age-matched control level after 3 mo (10% and 11% per fiber and millimeter, respectively, P < 0.05). The mean myonuclear domain levels in the control and unloaded groups were increased ~4.1 and ~2.5 times during the 3-mo postnatal development, respectively (Fig. 2C, P < 0.05). The level in the unloaded group was significantly less than the age-matched control at the end of 3-mo unloading (P < 0.05). Although the level in the control group was unchanged, that in the unloaded group increased to the control level within 1-mo recovery (P < 0.05).

Many compact myonuclei were noted in muscle fibers of control rats at the age of 3 mo (Fig. 3A). The volume of myonuclei in the control group was decreased during the 3-mo postnatal period (~43% vs. day 4 after birth, P < 0.05, Fig. 3B). However, the growth-associated reduction of myonuclear volume was inhibited by unloading. Larger myonuclei were observed in the unloaded than in control group (Fig. 3A). The mean volume at the end of 3-mo unloading was 35% greater than that of the age-matched control (P < 0.05). But the mean size became similar to that in controls within 1 mo of ambulation. The total DNA content in each myonucleus also decreased during the first 3 mo after birth (P < 0.05) but was stable thereafter (Fig. 3C). It was not influenced by unloading. The concentration of DNA (DNA content per cubic micrometer myonuclear volume) in the control group remained unchanged throughout the 6-mo experimental periods (Fig. 3D). However, it was significantly lower at the end of 3-mo unloading than the pre-unloading and the age-matched control levels (P < 0.05), although it was normalized within 1 mo.

The distribution of TUNEL-positive apoptotic myonuclei was generally minor in both control and unloaded groups at the age of 3 mo (Fig. 4). Although the percent distribution tended to be higher in the unloaded muscle fibers, there was no significant difference between the two groups (Fig. 4B).

Satellite cells. M-cadherin-positive (quiescent) satellite cells were not seen in muscle fibers at the 4th day after birth (Fig. 5, A and B). Although they were increased during 3 mo, the mean number of quiescent satellite cells per whole single fiber in the unloaded group (10.4) was significantly less than controls (46.3, P < 0.05). The number per millimeter fiber length was also less in the unloaded (1.1) than control muscle fibers (4.1, P < 0.05). Only BrdU-positive (mitotically active) satellite cells were observed in the fibers of 4-day-old rats (Fig. 5, C and D, and Fig. 6C). The total number of mitotically active satellite cells per whole fiber of control rats tended to decrease following growth (P > 0.05). That in the experimental group at the end of 3-mo unloading was significantly less than the pre-unloading level (~80%, P < 0.05) and the age-matched control (~75%, P < 0.05). However, it was gradually increased toward the control level during the 3-mo recovery periods. The number of mitotically active satellite cells per millimeter fiber length in the control group was decreased by 90% during 3 mo (0.3 vs. 2.9 at postnatal day 4, P < 0.05). Its decrease was further stimulated by unloading (0.1, ~97% vs. pre-unloading and ~69% vs. the age-matched control; P < 0.05). The number of these cells became identical to that in controls and remained stable during the 3-mo recovery periods.

The number of total (both M-cadherin and BrdU positive) satellite cells per whole fiber in the control group was increased by 16 times during 3 mo (P < 0.05, Fig. 6A). The growth-related increase of these levels was inhibited by unloading (~77% vs. the age-matched control; P < 0.05). It was increased in response to ambulation recovery gradually and reached the control level within 3 mo. The number of total satellite cells per millimeter fiber length in the control group did not change significantly during the 6-mo experimental period (Fig. 6B), and that in the unloaded group was decreased (~69% vs. pre-unloading, P < 0.05). It was 74% less than that

Fig. 4: A: typical distribution pattern of apoptotic myonuclei (arrowhead) labeled using terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) method in control and unloaded fibers at the end of 3-mo cage housing and hindlimb suspension, respectively. B: percent distribution of apoptotic myonuclei relative to the total myonuclei. Values are means ± SE; n = 4 for each group.
in the age-matched control also \( (P < 0.05) \). However, the number was gradually increased toward the control level and was normalized within 3 mo. The percentage of mitotically active satellite cells relative to the total cells at postnatal day 4 was 100\% (Fig. 6C). But the percentage in the control group dropped to \( \sim 12\% \) after 3 mo \( (P < 0.05) \) and tended to decrease further thereafter \( (P > 0.05) \). The level was not influenced by hindlimb unloading.

**DISCUSSION**

Rat soleus, which is known as an antigravity muscle and is predominantly composed of slow-twitch fibers, is ontogenetically developed at 1-G environment \( (6, 20, 31) \). However, the normal growth is inhibited by gravitational unloading \( (31) \). The most common ankle position during gravitational unloading of rat is a marked plantarflexion of ankle joints \( (18, 19, 28, 30, 33, 39) \). Both mechanical and neuronal activities of soleus muscle are inhibited in response to a plantarflexion of ankle joint, which shortens the whole length of soleus muscle, muscle fibers, and sarcomeres and, therefore, inhibits the tension production \( (18, 19, 28, 30) \). The soleus muscles of rats with mean body weight of \( \sim 300\) g developed \( \sim 44\) g and 16 g of passive tension when the anterior angle of ankle joints were maintained at \( \sim 30^\circ \) (simulated quadrupedal prone position) and \( \sim 90^\circ \) (bipedal standing position) on the floor \( (18) \). However, no tension was detected and the electromyogram activity was reduced \( \sim 88\% \) when the ankle joints were stretched during hindlimb unloading because of the passive shortening of muscle fibers and sarcomeres. The electromyogram activity was also inhibited in soleus muscle during unloading in the present study \( (29) \). Thus, it is clear that both electrical and mechanical activities of soleus were inhibited during the long-term unloading.

The current data showed that the unloading inhibited the adhesion of satellite cells, which then resulted in the failure of myonuclear accretion and of morphological (cross-sectional) growth of soleus muscle fibers. The hindlimb unloading was performed continuously between day 21 and month 3. However, the hindlimb-unloaded pups were returned to their mother for nursing for 1 h after every 5 h of unloading until postnatal day 21. The acute reloading during the nursing period might cause the muscle damage, which could activate the satellite cells and then myonuclear accretion. However, no growth-related myonuclear accretion was observed in soleus muscle fibers of pups that were unloaded using the same experimental protocol between postnatal day 4 and 21 in our previous study \( (31) \). Furthermore, the number of satellite cells in soleus muscle fibers of Wistar Hannover rats did not significantly change at the 2nd day of reloading after 16 days of unloading, which caused \( \sim 60\% \) decrease from the pre-unloading level \( (39) \). Therefore, the effects on the fiber properties by temporal loading during the nursing period might be minimal.

**Body and Muscle Weight**

The absolute weights of most of the organs and muscles, as well as the body weight, were less than those in the age-matched control immediately after the 3-mo unloading (unpublished observations). However, these weights relative to body weight were similar to those in the control. Therefore, inhibition of the growth-related increase of body weight, seen in the unloaded group, may be caused by reduction in the overall growth rate, not caused by restricted food intake since the same amount of food was supplied daily. It is speculated that the level of the nutrient absorbance might be inhibited in the unloaded rats. However, the data indicated that the growth of soleus muscle was further inhibited, maybe by local effect of unloading. Unloading effects on the mass in other plantarflexors, plantaris and both lateral and medial gastrocnemius, were minor (unpublished observations). Further, the percent weights of dorsiflexors, tibialis anterior, and extensor digitorum longus, relative to body weight in the unloaded group, were identical to those in the controls.

**Fiber Number**

Total fiber number in the unloaded soleus was identical to that in the age-matched controls \( (Fig. 1B) \). Both electrical and...
though the fiber CSA was significantly less (Fig. 1A) mainly due to an inhibition of cross-sectional growth. Al-buminhibited the growth-related increase of fiber mass. This was suggested that the increase in fiber length may be, in part, related to the load caused by the passive stretch due to the longitudinal growth of bone.

But it is not clear why the cross-sectional growth was not stimulated in the same muscle fibers. Although the fiber CSA was increased even in the unloaded soleus muscle fibers, the magnitude of increase was only 31% of the controls. It is suggested that the key factor(s) for the regulation of longitudinal and cross-sectional growth of muscle fibers is different. The reloading stimulated the expansion of fiber CSA. It is reported that tension development and afferent input play an important role in the maintenance of muscle fiber properties (18, 19). Passive shortening of soleus muscle due to plantarflexion of ankle joint during hindlimb unloading (18) or exposure to microgravity (19) inhibits the levels of tension development and afferent neurogram recorded at the L5 segmental level of spinal cord. It was also reported that the unloading-related shortening of sarcomere length was noted at the central region of soleus muscle fibers (39). The mean sarcomere length was 2.1 μm, which inhibits the in vivo tension development (18). Furthermore, the number of satellite cells in this region was clearly decreased. On the contrary, fiber atrophy is prevented even during hindlimb unloading, if the muscle is stretched by fixing the ankle joint at a dorsiflexed position using plaster cast (32). It is suggested that the gravitational load, which makes it possible to develop tension and maintain the afferent input and activity of satellite cells, is essential for the fiber hypertrophy in soleus muscle.

Myonuclei
Myonuclear accretion during the developing period plays an important role in the growth of muscle fibers (26, 31). The number of myonuclei in a single muscle fiber is increased in response to growth and development, as was reported previously (31). That in adult rats is, on the contrary, decreased in response to gravitational unloading during spaceflight (2), maybe due to apoptosis-related loss (1). The myonuclear number increased even during hindlimb unloading, although the degree of the increase was very low relative to that in the cage control. The changes in the myonuclear number, domain, and fiber CSA in response to unloading or growth were positively correlated with one another, as was previously reported (31), suggesting that the growth inhibition by unloading may be related to the suppression of the myonuclear accretion.

Furthermore, there is no significant difference in the percent distribution of TUNEL-positive apoptotic myonuclei between control and unloaded soleus muscle fibers (Fig. 4B). Although the growth-associated increase of myonuclear number was inhibited by unloading (40% vs. 3-mo old control), the number was significantly increased (40 times vs. 4-day). Thus, these results clearly indicated that the inhibition of myonuclear accretion, not apoptosis-related loss of myonuclei which was
observed in unloaded adult rat muscle (1), was the major cause of the smaller number of myonuclei in the unloaded group.

The size of myonuclei was decreased following the postnatal growth of control rats (Fig. 3B). However, the growth-associated reduction of myonuclear size was inhibited by unloading. Many larger myonuclei were noted in the unloaded fibers. These myonuclei generally contain the same total amount (Fig. 3C), but low concentration (Fig. 3D), of DNA compared with the small nuclei, although the functional differences between the small and large myonuclei are unclear. An increase in myonuclear size may have some relation with the decrease of myonuclear domain. The increase of the myonuclear size and decrease of DNA concentration were also seen in atrophied soleus muscle fibers of adult rats with reduced mechanical activity following 16 days of hindlimb unloading (39). Thus, the data in the current study may suggest that small myonuclei with greater concentration of DNA may have a superior capacity for protein synthesis than myonuclei with larger mass and lower DNA concentration. Although the key factor for the regulation of myonuclear mass is unclear, the data suggested that the chronic unloading inhibited the myonuclear function. Myonuclear domain and size were quickly improved in response to reloading (Figs. 2B and 3B), suggesting that they are also closely associated with the mechanical load applied to the muscle.

**Satellite Cells**

A satellite cell is known as a muscle precursor cell differentiated from a hematopoietic stem cell (4, 21, 36). A satellite cell proliferates and incorporates into a muscle fiber, and nuclei of satellite cells become myonuclei. The number of mitotically active satellite cells per unit number of fiber nuclei, including satellite cells and myonuclei, has been measured to estimate the mitotic activity of satellite cells (10, 34). All of the satellite cells at postnatal day 4 were mitotically active (Fig. 5, B and D, and Fig. 6C). According to the report by Schultz et al. (34), the number of mitotically active satellite cells decreases following growth. The number of mitotically active cells in the 3-mo-old control rats in the current study (3,3) was also insignificantly less than that in day 4 (4,2, Fig. 5C). The number of mitotically active satellite cells in soleus muscle fibers was further reduced by unloading with unknown mechanism. Schultz et al. (34) also reported that it was decreased following 1-wk hindlimb unloading. These findings suggest that hindlimb unloading causes suppression of satellite cell mitotic activity. Wang et al. (39) reported that the decreased number of both mitotically active and quiescent satellite cells was closely related to the reduction of mechanical stress caused by passive shortening of sarcomere. The length of muscle fibers and total number of sarcomeres were not affected by unloading in the present study. However, the activity of electromyogram was inhibited. Therefore, inhibition of growth-related increase of satellite cell number, seen in the unloaded group, may be caused by subnormal neural and/or mechanical activities.

Although the number of quiescent satellite cells in the control group was increased during the first 3 mo, the growth-associated increase was significantly inhibited by unloading (Fig. 5, A and B). The mitotically active-total satellite cells ratio in both groups at the age of 3 mo was only 12% of that observed 4 days after birth (Fig. 6C). And the number of total satellite cells in a whole muscle fiber of the unloaded group was only 23% of that in the age-matched control group (Fig. 6A). Thus, it is obvious that both satellite cell adhesion and proliferation were inhibited by chronic hindlimb unloading. These phenomena may inhibit the growth-associated increase of myonuclear number and then fiber size.

The number of satellite cells gradually increased in response to reloading without changing the mitotic ratio (Figs. 5C and 6C). Previous studies reported that the number of BrdU-positive satellite cells increased to the level more than the control during acute phase of the recovery from the muscular atrophy (26) and injury (35). Acute reloading immediately after the termination of 3-mo unloading may cause fiber damage, which may activate the satellite cells. It was also speculated that the mitotically active-total satellite cells ratio might be decreased during unloading. The decrease of mitotically active satellite cells from ~4 (day 4) to ~1 per fiber (month 3) in the unloaded group (Fig. 5C) might be related to a loss caused by apoptosis. However, this ratio remained stable in muscle fibers sampled at the end of unloading and 1–3 mo after reloading in the present study. Although the precise mechanism responsible is still unclear, it is interesting that the
mitotically active/total satellite cell ratio, which was decreased from 100% (day 4) to ~12% (month 3) in the control, was influenced by neither unloading nor reloading.

Quiescent satellite cells express several markers, including M-cadherin (16), Myf5 (4), myocyte nuclear factor (12), CD34 (4), c-Met (8), and Pax7 (36). Zammit and Beauchamp (40) reported that Pax7 is essential for the specification of satellite cells, suggesting that its expression may reveal cells “upstream” of committed satellite cells. Wang et al. (39) reported that the ratio of “active” vs. “quiescent” cells remained constant in response to both unloading and reloading. Even though the role of the quiescent satellite cells is not clear yet, these results may suggest their important roles in the regulation of muscle fiber properties.

**Perspective**

Although it has been well documented that satellite cells provide the new myonuclei for growing fibers, the plasticity and response of the satellite cell population to different loading conditions are not clear. Satellite cells are activated when the muscle is overloaded (9) or injured (35). On the contrary, the proliferation and differentiation of satellite cells are inhibited, if the growing intact skeletal muscles are unloaded by tail suspension (10, 26, 27, 34, 39) or spinal isolation model (15, 16). Effects of hindlimb unloading on satellite cell activity in growing skeletal muscle fibers of 20-day-old rats were first described by Durr and Schultz (10). Effects of unloading in 28-day-old (26) and 5-wk-old (39) rats were also studied. However, the hindlimb unloading in these studies was performed after the lactation period. Therefore, the responses of antigravity muscle fibers to unloading initiated in animals before completion of muscle fiber formation and acquisition of weight-supporting locomotion were investigated in the present study.

The summary of the major findings is shown in Fig. 7. The hypothesis that the growth of soleus muscle fiber during the developing period was load dependent was partially proved, and an important role of satellite cells in hypertrophy of muscle fibers during postnatal development was suggested. Although the number (39) and/or mitotic activity (10, 26, 27, 34) of satellite cells, as well as the fiber size and myonuclear number, is decreased when adult or weanling rats are unloaded, no reduction of these parameters was seen in the present study. The data showed that the unloading inhibited the adhesion of satellite cells, which then resulted in the failure of myonuclear accretion. As a result, the growth-associated hypertrophy of the fibers was inhibited. Thus, it is suggested that satellite cell recruitment, which is stimulated by gravitational loading, may play a key role in cross-sectional growth of muscle fibers. However, longitudinal growth of fibers was not inhibited even under the unloading condition in association with the longitudinal bone growth seen in the same rats of the current study (29). The increase of fiber number was not affected by unloading either, suggesting that the muscle cell formation may be genetically programmed or stimulated by stretching of fiber due to bone growth (29).

The fiber properties in the control rats were generally stable between postnatal month 3 and 6, indicating that the growth was completed during the first 3 mo of life. However, the inhibited growth in the unloaded group was recovered toward the control levels following the 3-mo reloading. It is indicated that the fibers were still able to adapt to the external load, even though most of the properties were not completely recovered within 3 mo to the levels of the age-matched controls. These recoveries were associated with the normalization of satellite cell profiles in response to reloading. It is suggested that the satellite cell-related stimulation in response to mechanical load and/or neural activity plays an essential role in the cross-sectional growth of soleus muscle fibers.

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