Role(s) of nucleoli and phosphorylation of ribosomal protein S6 and/or HSP27 in the regulation of muscle mass

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Kawano F, Matsuoka Y, Oke Y, Higo Y, Terada M, Wang XD, Nakai N, Fukuda H, Imajoh-Ohmi S, Ohira Y. Role(s) of nucleoli and phosphorylation of ribosomal protein S6 and/or HSP27 in the regulation of muscle mass. Am J Physiol Cell Physiol 293: C35–C44, 2007. First published December 20, 2006; doi:10.1152/ajpcell.00297.2006.—Effects of 14 days of hindlimb unloading or synergist ablation-related overloading with or without deafferentation on the fiber cross-sectional area, myonuclear number, size, and domain, the number of nucleoli in a single myonucleus, and the levels in the phosphorylation of the ribosomal protein S6 (S6) and 27-kDa heat shock protein (HSP27) were studied in rat soleus. Hypertrophy of fibers (+24%), associated with increased nucleolar number (from 1–2 to 3–5) within a myonucleus and myonuclear domain (+27%) compared with the preexperimental level, was induced by synergist ablation. Such phenomena were associated with increased levels of phosphorylated S6 (+84%) and HSP27 (+28%). Fiber atrophy (−52%), associated with decreased number (−31%) and domain size (−28%) of myonuclei and phosphorylation of S6 (−98%) and HSP27 (−63%), and with increased myonuclear size (+19%) and ubiquitination of myosin heavy chain (+33%, P > 0.05), was observed after unloading, which inhibited the mechanical load. Responses to deafferentation, which inhibited electromyogram level (−47%), were basically similar to those caused by hindlimb unloading, although the magnitudes were minor. The deafferentation-related responses were prevented and nucleolar number was even increased (+18%) by addition of synergist ablation, even though the integrated electromyogram level was still 30% less than controls. It is suggested that the load-dependent maintenance or upregulation of the nucleolar number and/or phosphorylation of S6 and HSP27 plays the important role(s) in the regulation of muscle mass. It was also indicated that such regulation was not necessarily associated with the neural activity.

rat soleus muscle; functional overload; deafferentation; 27-kDa heat shock protein; ubiquitination of myosin heavy chain

GRAVITATIONAL UNLOADING CAUSES a decrease in the fiber size of antigravity muscles, such as soleus in human (29) and rats (3, 26). Soleus muscle of rat is passively stretched and neurally active in vivo at the quadrupedal posture on the floor because of the dorsiflexion of the ankle joint (19, 20, 27). The loss of electromyogram (EMG) activity in soleus muscle and afferent neuromuscular recorded at the L5 segmental level of the spinal cord was noted, when the ankle joint was plantarflexed during exposure to an actual microgravity (20) or hindlimb suspension (19, 27). Furthermore, tension, measured by placing a force transducer at the distal tendon, was not detected in the soleus muscle when the ankle joint was maintained at a plantarflexed position, because of the short length of muscle fibers and sarcomeres (19). These results suggest that the gravitational unloading inhibits both the neural activity and mechanical load.

Previous studies (3, 36) reported that the loss of myonuclei was associated with the unloading-related atrophy of soleus muscle fibers. It was also reported that the protein synthesis activity was suppressed in the soleus muscle following the hindlimb unloading by tail suspension (10). Furthermore, the expression of the heat shock protein (HSP), which is a molecular chaperone, decreased by the unloading of muscle (14, 31). These results clearly indicate that load applied is an important factor for muscle plasticity. However, it is still unclear how the neural activity or mechanical load, applied to the muscle fibers, plays a role(s) in the regulation of muscle mass. Ohira (25) reported that no compensatory hypertrophy in response to tenotomy of the synergists was induced in rat soleus muscle when the afferent nerve was transected, suggesting that the afferent information plays a significant role in the regulation of muscle mass. However, the responses of the intramuscular parameters, such as the myonuclear number, protein synthesis activity, and heat shock response, to deafferentation are unknown.

Nucleolus is recognized as a loop of the ribosomal DNA region where the ribosomal RNA (rRNA) is transcribed by RNA polymerase I (7). The number of nucleoli reflects the transcriptional activity of rRNA, which leads to intracellular protein synthesis (9). It is well known that the ribosomal protein S6 (S6), which plays an important role in the initiation of the translation of messenger RNA, is phosphorylated following the activation of the Akt/mammalian target of rapamycin pathway (8, 21). Small HSPs, such as the 27-kDa HSP (HSP27), are abundantly expressed in the skeletal muscles, especially those comprised primarily of fibers containing slow type I myosin heavy chain (MHC) (14), and may also act as a molecular chaperone (33). Previous studies reported that phosphorylated HSP27 stabilized the actin cytoskeleton in skeletal muscle during cell stress (11, 23). It was also reported that unloading-related atrophy was accompanied by activation of the ubiquitin-proteasome pathway (17, 39), which is known as the major pathway responsible for proteolysis, including contractile proteins such as actin and myosin (6, 18). However, it is still unknown how these parameters, which may play major roles in the regulation of protein synthesis and/or degradation,
response to unloading or loading with or without afferent neural input.

Therefore, the current study was carried out to test the hypothesis that the level of mechanical loading associated with afferent input stimulates changes of muscle mass as well as the signaling pathways for protein translation and degradation. The effects of decreased muscle activity due to hindlimb suspension or increased activity caused by synergist ablation with or without deafferentation on the morphological characteristics of soleus muscle fibers of adult rats were investigated. Furthermore, the possible role of the intranuclear number of nucleoli was also studied.

**MATERIALS AND METHODS**

**Animal care and surgical procedures.** All experimental procedures were conducted in accordance with the Japanese and American Physiological Society’s “Guide for the Care and Use of Laboratory Animals.” This study was also approved by the Committee on Animal Care and Use at Osaka University. Twelve-week-old male Wistar rats (Nihon CLEA) with a mean body weight of ~340 g were randomly separated into pre- (n = 5) and postexperimental control (n = 8), synergist-ablated (n = 11), sham-operated control (n = 8), deafferentated (n = 8), synergist-ablated plus deafferentated (n = 8), and hindlimb-unloaded (n = 8) groups. The denervation model has been used to inhibit neural activity (16, 28, 30). However, denervation leads to a limb paralysis and plantarflexion-related passive shortening of ankle dorsiflexors that also inhibits the mechanical load to muscles. Furthermore, hindlimb unloading-related atrophy of soleus muscle was not advanced by additional treatment with denervation (28). Therefore, the group with denervation alone was not included in the present study.

The rats in the preexperimental control group were killed on the first day of the experiment following the methods shown below. All surgeries were performed under anesthesia with intraperitoneal (ip) injection of pentobarbital sodium (5 mg/100 g body wt). The distal tendons of the left plantaris and gastrocnemius muscles were completely transected to avoid reattachment of the tendons to the soleus muscle in the synergist-ablated group. The epimyscous connective tissues, as well as the nerve supply and blood flow, were kept intact. Therefore, mechanical interaction of the soleus with the surrounding muscles still exists, and shear forces on soleus could be exerted because of the tenotomy-related shortening of plantaris and gastrocnemius. As for deafferentation, a skin incision was made along the vertebral column at the L4–5 segmental levels was exposed, and the afferent fibers including dorsal root ganglions (DRGs) were transected (~5 mm). Both treatments were performed for the synergist-ablated plus deafferentated rats. Rats in the sham-operated control group had their left DRGs and roots exposed, but the nerve supply was kept intact. The contralateral side was kept intact. Fourteen days of ambulation were allowed after the surgery.

Rats in the hindlimb-unloaded group were tail-suspended as described previously (27). Briefly, a sticky tape (~3-cm width and 5-cm length) with good cushion was placed longitudinally on the dorsal and ventral sides of the midtail. These tapes were further surrounded cross-sectionally by a tape. The attachment of the tapes 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 plastic cage (30 × 30 cm and 30-cm height) at a height allowing the forelimbs to support the weight, yet prevent the hindlimbs from touching the floor or the wall of the cage. The rats could reach the food and water freely by using their forelimbs. Such treatments were performed for 14 days. Each rat was housed in a cage individually. A commercial solid diet (CE-2, Nihon CLEA) and water were supplied ad libitum. Temperature and humidity in the animal room, with a 12:12-h light-dark cycle, were maintained at ~23°C and ~55%, respectively.

On the 14th day after surgery or start of the suspension, sampling of the soleus muscle was performed (n = 5 for each group) under anesthesia with ip injection of pentobarbital sodium (5 mg/100 g body wt). The left soleus muscle was isolated from each rat after the cardiac excision, which was performed to reduce the blood proteins within the soleus. The muscle was subsequently cleaned of excess fat and connective tissue, weighed, frozen in liquid nitrogen, and stored at −80°C until the analyses. Right muscle was not used for any analyses.

**EMG recordings.** The remaining rats in the postexperimental control, synergist-ablated, hindlimb-unloaded, sham-operated control, deafferentated, and synergist-ablated plus deafferentated groups were used for analysis of EMG activity (n = 3 for each group). Implantation of electrodes was performed immediately after the completion of each treatment, as described previously (19, 20, 27). Briefly, the rat was anesthetized with ip injection of pentobarbital sodium (5 mg/100 g body wt), and then a skin incision was made along the sagittal suture of the skull after shaving and cleaning with 100% ethanol. The exposed skull was dried, and a 2.5-mm-diameter burr hole was firmly anchored to the skull using both screws and dental cement. Three enamel-coated constantan wires, 80 μm in diameter, were led subcutaneously from the connector to the back region and left hindlimb.

The left soleus muscle was exposed, keeping the blood and nerve supplies intact. Bipolar electrodes were implanted into the muscle. The wires were inserted by threading them individually through a 27-gauge hypodermic needle being passed through the muscle individually. The needle was carefully withdrawn, and the insulated wire was stripped (~0.5 mm). The section of the wire, with the insulation removed, was implanted into the midbelly of the muscle. Two wires were inserted in parallel with the muscle fibers (~2 mm apart). Each wire was secured with a suture at its entry and exit from the muscle, so that the stripped portion of the wire in the muscle was fixed.

After a 1-day recovery from surgery, soleus EMG in conscious rats was recorded at quadrupedal posture on the floor or during hindlimb suspension for ~1 h. The EMG recordings (~1 h/day) were performed during the rest of the 14-day experimental period. The electrical signals were amplified (~1,000) and recorded on a digital audio recorder (PC216AX, Sony) at 2.5 kHz. The amplified raw signals stored in the cassette tape recorder, in which the 60-Hz signals were filtered, were processed by PowerLab/16sp (ML795, AD Instruments), an analog-to-digital (A/D) converter, digitized at 2 kHz, and were stored on a hard disk. The total integrated area of EMG was determined using a computer software package (Chart v4.0.1, AD Instruments). The total mean integrated EMG activity per hour was calculated (20). After the recording, the rats used for the EMG recording were killed by an overdose of pentobarbital sodium.

**Tension recording.** Using three rats, we measured the tension development in soleus muscle. The rats were anesthetized with ip injection of pentobarbital sodium (5 mg/100 g body wt), and the left soleus muscle was carefully exposed. A force transducer made of silver buckle with strain gage was placed at the distal tendon of soleus muscle. After the rats recovered from the anesthesia, the development of in vivo tension was recorded at rest on the floor and during tail suspension and quadrupedal walking, using a strain meter (PCD-30A: Kyowa, Tokyo, Japan) calibrated using an isometric force transducer (TB-654T; Nihon Kohden, Tokyo, Japan). The strain was set to zero when the ankle joint was plantarflexed and soleus muscle was passively shortened in response to hindlimb suspension. After the recording, the rats were again anesthetized and the distal tendons of the left plantaris and gastrocnemius muscles were transected. The recording of tension development was performed again the next day in rats at rest and during quadrupedal walking on the floor.

**Muscle preparation.** Histochemical analyses in the longitudinal segment of the muscle fibers and analyses of protein expression levels in the muscle homogenates were performed. The frozen muscle was cross-sectionally cut at the midbelly region into two sections. The
proximal one-half of the sections was gradually thawed to room temperature in a low–cold-crem relaxing solution as described previously (4). Single fiber segments (at least n = 60 from each muscle) were mechanically isolated by using fine tweezers under a dissecting microscope, placed on a gelatin-coated glass slide, air-dried, and stored at −5°C until analyses. Mechanical isolation of single fibers has been shown to strip off the basal lamina of muscle fiber including satellite cells (4). The remaining distal portion of muscle was homogenized in the ice-cooled cytoplasmic protein fractionation solution (Bio Vision) using a glass homogenizer. The homogenate was centrifuged at 12,000 g for 5 min, and the supernatant was isolated and stored at −80°C until analyses of S6, ubiquitinated MHC, and HSP27 expression. We use HSP27 to refer to murine HSP27, which is a protein homologous to human HSP27 (10).

Determination of the fiber properties. The single muscle fiber segments were thawed and dried at room temperature. In one-half of the fibers (n = 30 for each muscle), the myonuclei were stained with 1.5 × 10−7 M propidium iodide diluted in phosphate-buffered saline for 5 min. In the other fibers (n = 30 for each muscle), the nucleoli were silver-stained using a modified method described previously (32). Briefly, fiber segments were fixed in 10% buffered formalin for 5 min and submerged in silver solution containing 17% AgNO3, 1% formic acid, and 1% gelatin for 30 min. Subsequently, the myonuclei were stained with hematoxylin for 5 min. A FV-300 confocal microscope with an argon laser (488 nm peak wavelength, Olympus) was used to analyze the fiber cross-sectional area (CSA), the number and CSA of the myonuclei, and the distribution of the nucleoli. First, a maximum-intensity projection rotated orthogonally to the long axis of the fiber was produced, and the fiber CSA was measured at three nonoverlapping regions randomly chosen along the fiber length. The number of myonuclei in the single fiber segment was counted. The length of the fiber segment was also measured. Myonuclear domain (5, 12) was calculated by multiplying the fiber CSA and length of the fiber segment and dividing by myonuclear number. The fiber CSA and the length of fiber segment were normalized at a 2.5-μm sarcomere length. The myonuclear number per millimeter of fiber length was calculated. The myonuclear size and the number of nucleoli were also measured in each myonucleus by Nomarski optic scan. The nucleoli, myonucleoplasm, and fiber cytoplasm were clearly separated by the light penetration ratio because of the staining intensity. To measure the myonuclear size, the outline of the myonucleus was enclosed in the scanned image and the area was measured. At least 50 myonuclei were checked for each fiber. Damaged regions and excessively stretched (sarcomere length >3.5 μm) regions were omitted from the above analyses.

SDS-PAGE for analyses of protein expressions. The cytoplasmic extraction of the soleus muscle was dissolved in equal amounts of 2× SDS sample buffer (20% glycerol, 12% 2-mercaptoethanol, 4% SDS, 100 mM Tris·HCl, 0.05% bromophenol blue, pH 6.7) and adjusted at a final concentration of 0.1 (for HSP27) or 2 μg protein/μl (for S6 and ubiquitinated MHC) by 1× SDS sample buffer. SDS-PAGE was carried out on 7.5% (for ubiquitinated MHC) or 10% (for S6 and HSP27) polyacrylamide slab gel at a constant voltage of 40 V for 20 h at 4°C. Equal amounts of protein (10 μl) were loaded on each lane. The protein extracts of five muscles from the preexperimental control rats were combined, and the mixture was loaded as the control for all gels.

Western blot. Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes (0.2-μm pore size, Bio-Rad) by using the trans-blot cell (Bio-Rad) at a constant voltage of 60 V for 2 h at 4°C. After the transfer of protein, the membranes were blocked in the blocking buffer [5% blocking reagent (Amersham Biosciences) with 0.1% Tween 20 in Tris-buffered saline (TBSB)] for 1 h. The membranes were incubated overnight at 4°C with anti-S6 phosphorylated at Ser235/236 (1:1,000, Cell Signaling Technology), anti-ubiquitin (1:2,000, Sigma-Aldrich), anti-HSP27 (1:2,000, Stressgen), anti-HSP27 phosphorylated at Ser85 (1:2,000, Abcam), and antibody diluted in TTBS containing 5% bovine serum albumin (BSA). Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody to rabbit or mouse immunoglobulin G (Cell Signaling Technology) for 30 min. The antibody-bound protein was detected by a chemiluminescence method using an enhanced chemiluminescence plus kit (Amersham Biosciences).

Quantification of the bands was performed using computerized densitometry. The protein level was expressed as integrated density, which was calculated as the mean density × the band area. As for the analysis of the ubiquitinated MHC, the integrated density was measured in the bands between 200 and 250 kDa. After the analyses, the membranes were incubated in stripping solution (Nacalai Tesque) for 15 min to strip the antibodies. The membranes were blocked again and incubated overnight at 4°C with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon) diluted in TTBS containing 5% BSA. Blots were incubated with HRP-conjugated secondary antibody to mouse immunoglobulin G (Amersham Biosciences) for 30 min. The detection of the bands or spots was followed by the above procedures. The level of protein expression was normalized by the integrated density of the respective GAPDH band or spots and was expressed as the value relative (%) to the mean preexperimental control level (100%).

Statistical analysis. All values are expressed as means ± SE. Significant differences were examined by repeated measures of ANOVA followed by Scheffe’s post hoc test. Differences were considered significant at the 0.05 level of confidence.

RESULTS

EMG activity and force production. The EMGs in the soleus muscle of postexperimental control rats were tonic generally when the rats were sedentary on the floor, although greater bursts were noted when the rats were in motion. The mean integrated EMG level tended to increase 1 day after the ablation of synergists (Fig. 1A, P > 0.05). In contrast, the EMG levels were significantly lowered by hindlimb unloading (−86%) and deafferentation with (−65%) or without synergist ablation (−61%) relative to cage controls. The mean EMG levels in the group with synergist ablation gradually decreased, and those in the hindlimb-unloaded and deafferentated groups increased toward control levels, especially after 1 wk (data not shown). Those in the groups with synergist ablation and hindlimb unloading reached the control level 14 days later. However, the levels in the groups with deafferentation with (−32%) or without (−46%) synergist ablation were still less than those of the postexperimental and sham-operated control groups (P < 0.05).

Force, which was set to zero when the ankle joint was plantarflexed and soleus muscle was passively shortened in response to hindlimb suspension, was constantly generated in the soleus muscle at rest in a quadrupedal posture on the floor (≈44 g) (Fig. 1B). Force was elevated in response to synergist ablation (+102% vs. preexperimental control, P < 0.05). The levels of force production during walking in both normal control and synergist-ablated groups were further increased (+130 and +71% vs. at rest on the floor, respectively).

Muscle weight. Absolute weight of the soleus muscle was 9% greater in the postexperimental group than the preexperimental control because of growth during the experimental period (P < 0.05), although the weight relative to body weight was stable (Fig. 2). The absolute and relative weights were further increased (+32 and +23% vs. preexperimental control, respectively) by the ablation of synergists (P < 0.05) but were decreased (−49 and −38%, respectively) following hindlimb
unloading (P/H11021 0.05). A significant decrease in the absolute (12%) and relative (12%) muscle weights, compared with preexperimental control rats, was also induced by deafferentation. The deafferentation-associated decrease in absolute and relative weights of the soleus muscle was prevented by additional treatment with the ablation of synergists, and the weight was identical to control levels. Muscle weight was not influenced by the sham operation. Weight in the hindlimb-unloaded group was less than in any other groups.

Fiber size and myonuclei. Mean fiber CSA in the postexperimental control rats was greater (+8%) than that in the preexperimental controls (P < 0.05, Fig. 3A). A further increase in the fiber CSA was induced following 2 wk of synergist ablation (+24% vs. preexperimental control, P < 0.05). In contrast, fiber CSA in the hindlimb-unloaded rats was decreased to approximately one-half of that in the preexperimental control group (P < 0.05). A significant decrease in the fiber CSA (−27%) was also seen in the deafferentated rats, whereas the fiber CSA in the synergist-ablated plus deafferentated rats did not change from the preexperimental control level. Fiber CSA was not affected by the sham operation.

The myonuclear number per millimeter of fiber length was significantly increased in the postexperimental control compared with the preexperimental control rats (+16%, P < 0.05, Fig. 3B). Although the loss of myonuclei was noted in the hindlimb-unloaded rats (−31% vs. preexperimental control, P < 0.05), the ablation of synergists, sham operation, deafferentation, and ablation of synergists plus deafferentation did not affect the myonuclear number. The myonuclear domain size was identical in all control groups (pre- and postexperimental and sham-operated control, Fig. 3C). The domain size in the synergist-ablated group was significantly greater than that of pre- (+27%) and postexperimental (+30%) control rats, whereas that in the hindlimb-unloaded (−28 and −26%) and deafferentated group (−22 and −20%) was less than in the pre- and postexperimental controls, respectively (P < 0.05). The deafferentation-related decrease of myonuclear domain size was prevented by the additional treatment with the ablation of synergists in synergist-ablated plus deafferentated rats.

Myonuclei containing different numbers of nucleoli were observed (Fig. 4, A and B). The mean CSA of the single myonucleus was identical among the control groups (pre- and postexperimental and sham-operated control, Fig. 4C). The size was not affected by the ablation of synergists or deafferentation but was increased in the hindlimb-unloaded (+19%) and synergist-ablated plus deafferentated (+10%) rats (vs. preexperimental control, P < 0.05). The mean number of nucleoli in the single myonucleus of the pre- and postexperimental and sham-operated control groups was 1.6–1.7, and the number was increased by the ablation of synergists (2.1 ± 0.03) and the ablation of synergists plus deafferentation (2.0 ± 0.03).
The remaining 46–50%, 6–9%, and <1% of myonuclei contained two, three, and four nucleoli, respectively. The distribution of myonuclei containing a single nucleolus was decreased by the ablation of synergists (−24%) and the ablation of synergists plus deafferentation (−19%), while myonuclei containing three (+34 and +24%, respectively) and four (+7 and +4%, respectively) nucleoli was increased (vs. preexperimental control, $P < 0.05$). Myonuclei containing even five nucleoli were observed in the synergist-ablated (0.4%) and synergist-ablated plus deafferentated (0.4%) groups.

Expression of S6, HSP27, and ubiquitinated MHC. The expression level of S6 phosphorylated at Ser235/236 in the postexperimental and sham-operated control groups was identical to the level in the preexperimental controls (Fig. 5). The level of phosphorylated S6 in the synergist-ablated group was elevated compared with the pre- and postexperimental controls (+84%, $P < 0.05$). The level in the group with hindlimb unloading (−98%) or deafferentation alone (−64%) was significantly less than in the age-matched postexperimental and sham-operated control groups, however ($P < 0.05$). The deafferentation-related decrease of phosphorylated S6 was increased toward the control level by the addition of synergists.

The expression level of ubiquitinated MHC in the postexperimental and sham-operated control groups was also identical to the level in the preexperimental controls (Fig. 6). The ablation of synergists did not affect the expression level. However, the expression level of the ubiquitinated MHC in the hindlimb-unloaded (+33%) and deafferentated (+29%) groups tended to be increased ($P > 0.05$). The deafferentation-related increase of the ubiquitinated MHC was normalized by addition of synergist ablation (−31% vs. deafferentated group, $P > 0.05$).

No significant responses were observed in the expression level of total HSP27 in all groups (Fig. 7A). However, the phosphorylated level of HSP27 in the synergist-ablated group was greater compared with the pre- and postexperimental controls (+28%, $P < 0.05$, Fig. 7B). In contrast, the level was less in the hindlimb-unloaded (−63%) and deafferentated (−28%) groups ($P < 0.05$). The deafferentation-related decrease of phosphorylated HSP27 was prevented by additional treatment with the ablation of synergists.

DISCUSSION

Effects of hindlimb unloading or loading by ablation of synergists with or without deafferentation on the characteristics of fibers and the expression of S6, HSP27, and ubiquitinated MHC in the soleus muscle were studied in adult rats to investigate the role(s) of the mechanical load with or without afferent input in the regulation of muscle mass. To our knowledge, this is the first study showing that the muscle mass is maintained or elevated if the number of nucleoli and phosphorylation of S6 and HSP27 are increased in response to enhanced mechanical stimuli, regardless of the afferent neural input.

Mechanical load and/or neural activity. Generally, the data agree with previous studies reporting that hindlimb unloading caused atrophy (3, 26) and ablation of synergists induced compensatory hypertrophy (34, 35). These observations clearly indicated that soleus muscle mass is regulated by mechanical load and neural activity, because both mechanical load and neural activity are inhibited by hindlimb unloading (19, 20) and stimulated by ablation of synergists (15, 19, 34). The force production of soleus, measured in vivo, was also increased following synergist ablation in the present study. Roy et al. (34) reported that the increases of maximum isometric twitch and tetanic tension in the remaining musculature was noted 12–14 wk after the ablation of synergists, clearly indicating
that the compensatory adaptation in response to the ablation of the synergists resulted from functional overload-associated activation of the muscle.

Huijing and Jaspers (15) reported that the active force exerted in the remaining muscle was increased when muscle length of the synergists was shortened, and also suggested that the myofascial force transmission from the adjacent muscles might contribute to the moment of force production. Since extramuscular connective tissues were not dissected, shear forces on the soleus muscle also could be exerted because of the shortening of tenotomized gastrocnemius and plantaris in the present study. Furthermore, it was reported that muscle hypertrophy caused by functional overload was associated with increased insulin-like growth factor I, which has anabolic effects (1). Therefore, known and/or unknown factors, other than the mechanical load, may also play some role(s) in the induction of hypertrophy caused by synergist ablation.

As for the neural activity, the integrated EMG activities of soleus with synergist ablation tended to be elevated at a quadrupedal posture on the floor relative to those of controls 1 day after the surgery ($P > 0.05$). However, those levels at the 14th day were identical to that of the normal controls. It was also reported that the integrated level of the soleus EMG and efferent neural activities recorded at rest on the floor were not drastically increased 1 day after the ablation of synergists, although the afferent neural activity was increased 1.7-fold over the preexperimental level (19). A similar phenomenon was observed when rats were exposed to 2-G (20). The mean integrated soleus EMG activities of conscious rats were increased when gravity was acutely elevated from 1-G to 2-G,
gradually, during the ascending phase of a parabolic flight of a jet airplane. However, those activities recorded 1–10 days after exposure to 2-G, created by the centrifugation of animal centrifuge, were identical to those at rest on the floor in the 1-G environment, suggesting that EMG is maintained at the 1-G level if the rats maintain the sedentary posture, being accustomed to chronic exposure to a new environment (X. D. Wang et al., unpublished observation). Force production of soleus was, of course, greater during walking than at rest on the floor (Fig. 1B). It was further increased following the synergist ablation, although the elevation of EMG level was not statistically significant (Fig. 1A). These results suggested that the EMG level is not necessarily directly associated with the load applied to the muscle.

In contrast, the EMG activities of soleus during hindlimb suspension and at a quadrupedal posture on the floor 1 day after deafferentation with or without synergist ablation were decreased. These activities remained low during the first week, although they were gradually increased toward the control levels. The mean activity during suspension, with intact neural innervation, returned to the control level after 14 days (Fig. 1A). It was also reported that the integrated level of the soleus EMG and both afferent and efferent neural activities were decreased in response to hindlimb unloading but were normalized within 2 wk, maybe because of the sarcomere reorganization (19). However, it was suggested that the activities in the deafferentated groups with and without synergist ablation were still 46 and 32% less than in the controls, respectively (Fig. 1).

Muscle mass regulation by mechanical or neural factors

Fig. 5. Percent changes in phosphorylated ribosomal protein S6 (S6) expression relative to the mean preexperimental control level (100%). The typical expression patterns of the bands are also shown. Values are means ± SE; n = 5 in each group. P < 0.05 vs. postexperimental control (+), sham-operated control (†), and deafferentated (§) groups.

Fig. 6. Percent changes in ubiquitinated myosin heavy chain (MHC) expression relative to the mean preexperimental control level (100%). Values are means ± SE; n = 5 in each group. §P < 0.05 vs. deafferentated group.

Fig. 7. Percent changes in total (A) and phosphorylated (B) 27-kDa heat shock protein (HSP27) expression relative to the mean preexperimental control level (100%). The typical expression patterns of the bands are also shown. Values are means ± SE; n = 5 in each group. P < 0.05 vs. postexperimental control (+), sham-operated control (†), and deafferentated (§) groups.
remodeling of sarcomere, in which total number was decreased and the interval between each Z-line was increased toward the normal level, was induced, and EMG level was also normalized during 2 wk of hindlimb unloading (19). However, the in vivo length of the sarcomeres, especially at the central region of muscle fibers, was not completely recovered (19, 36). And the atrophy of fibers and decrease of myonuclear number were still progressed (27, 36, 38). It is again suggested that the neural activity estimated by EMG recording is not closely related to the morphological responses of muscle to short-term (~2 wk) unloading.

**Myonuclear properties.** In the hindlimb-unloaded rats, the fiber CSA, as well as the number of myonuclei and myonuclear domain size, was decreased, suggesting that the capability of protein synthesis in the myonucleus was possibly downregulated by hindlimb unloading. Allen et al. (5) reported that myonuclear apoptosis, which may cause the loss of myonuclei, was noted in the soleus muscle fibers on the third day after the start of hindlimb unloading when the EMGs were inhibited (19, 27). But it is unclear at which time point the loss of myonuclei was initiated.

Previous studies (3, 29, 36) also reported that decreases of myonuclear number and domain size were seen in soleus muscle fibers atrophied following unloading, whereas myonuclear number in fibers atrophied by deafferentation remained unchanged in the present study. Although deafferentation inhibits neural feedback, muscle strain might be maintained, because normal posture maintenance keeping the ankle joints at a dorsiflexed position on the floor was possible. The rats were also capable of performing voluntary locomotion. Thus it is clear that the fiber atrophy seen in the group treated with deafferentation, which also caused a reduced integrated EMG activity, is not directly related to the mechanical unloading seen in the hindlimb-suspended group (19, 25, 27). It is speculated that deafferentation-related inhibition of the feedback mechanism may play some role in the induction of fiber atrophy, as well as the inhibition of phosphorylation of S6 and HSP27, as stated below.

The unloading-related increase of myonuclear size was noted, as was reported previously (36). Myonuclear size was also increased in the group with the ablation of synergists plus deafferentation, although it was not influenced by a single treatment of synergist ablation or deafferentation. The mechanisms underlying the change in myonuclear size are still unclear. But the data suggest that the increase in myonuclear size may not be a beneficial phenomenon in terms of protein synthesis (36).

The number of nucleoli, which reflects the rate of rRNA production, is closely associated with the stage of the cell cycle (9). It was suggested that the nucleolar number in the quiescent or differentiated cells was less, because all of the nucleii in the myotubes were positive for p21, the G_{0/1} arresting factor (24, 40). We also observed that the nucleolar number was drastically reduced in response to in vitro differentiation from myoblasts to myotubes (Y. Matsuoka et al., unpublished observation). Furthermore, the number in myoblasts was significantly less if the creatine analog β-guanidinopropionic acid, which decreases the level of intracellular ATP (13), was supplemented in the culture medium. These results suggest the existence of cell cycle-independent signals in the regulation of nucleolar properties. However, the precise mechanism responsible for the regulation of nucleoli in the skeletal muscle fibers in vivo is still unclear.

The mean number of nucleoli within a single nucleus of the controls was one to two, but it was increased to three, four, or five in response to the ablation of synergists, suggesting that the level of rRNA production in the myonucleus (9) was elevated in response to enhanced mechanical load. The number was also increased by the ablation of synergists plus deafferentation, even though the neural activity was suppressed by deafferentation. The number of nucleoli was not influenced by deafferentation. Therefore, it was suggested that the mechanical load could be one of the major stimuli that enhance the intramuscular capacity for protein synthesis via increased number of nucleoli, even though the cell cycle in the muscle fibers may be still arrested. These results suggested that the nucleus containing larger numbers of nucleoli may have a higher protein synthesis activity. Therefore, it is clear that the mechanical load applied to muscle fibers plays an important role in the regulation of nuclear morphologies, which probably affects the intramuscular protein synthesis.

**Protein expression.** The level of S6 phosphorylation in soleus was increased following the ablation of synergists in the present study, suggesting that protein synthesis in the soleus muscle may be stimulated by an elevated level of mechanical load and/or afferent input. In contrast, the level of S6 phosphorylation was lowered by hindlimb unloading and deafferentation, which caused a significant atrophy of whole muscle and muscle fibers, probably due to inhibited protein synthesis. However, the lowered level of phosphorylated S6 in the deafferentated rats, with some mechanical load was maintained because the maintenance of normal posture keeping the ankle joints at a dorsiflexed position on the floor, was still ~18-fold greater than that in hindlimb-unloaded rats (Fig. 5). Furthermore, application of the extra mechanical load by addition of synergist ablation in the deafferentated rats prevented the severe loss of S6 phosphorylation induced by deafferentation. These results suggest that the mechanical load plays a crucial role in the intramuscular protein synthesis.

HSP27 is highly expressed in muscle predominantly composed of slow fibers, such as soleus and adductor longus muscle (14), and is also known as a molecular chaperone (33). Several cell stressors, such as hypoxia, hyperthermia, and mechanical stress, stimulate the expression of the HSP27 (14, 22, 37). In the present study, the total amount of HSP27 expression was not changed by any experimental model, although it was reported that the expression level was decreased following spinal isolation because of hindlimb inactivation (14). It is reported that phosphorylated HSP27 plays a role in sarcomere reorganization (11, 23). We also observed that the hindlimb unloading-associated decrease of phosphorylated HSP27 was induced particularly in the myofibrillar fraction of soleus muscle homogenates, suggesting that the lowered phosphorylation of HSP27 might have some relationship to the breakdown of myofibrillar proteins in atrophied muscle (F. Kawano et al., unpublished observation). Furthermore, recovery from unloading-induced atrophy was accompanied by an increase in phosphorylated HSP27 expression, and muscle did not recover from atrophy if an inhibitor peptide for phosphorylation of HSP27 was intramuscularly injected before the start of reloading, suggesting that phosphorylated HSP27 may play an important role in the regulation of muscle mass. Although
the precise mechanisms underlying phosphorylation and translocation of HSP27 in vivo are still unknown, phosphorylation of HSP27 might be necessary for cytoskeletal organization, which could regulate the skeletal muscle mass. In the present study, the phosphorylation level in HSP27 was inhibited by hindlimb unloading or deafferentation. However, the decrease in phosphorylation of HSP27 was more than twofold in the hindlimb-unloading compared with the deafferentated group; this may be due to inhibition of mechanical load. Therefore, loss of mechanical load and neural activity might inhibit intramuscular cytoskeletal organization and cause muscle fiber atrophy.

In contrast, the increased phosphorylation of HSP27 following ablation of synergists may be closely related to the enhanced mechanical load. Although HSP27 expression in the myofibril fraction was not determined in the present study, the data could suggest that cytoskeletal organization may be promoted in soleus muscle fibers following the ablation of synergists. Treatment with the ablation of synergists in addition to deafferentation prevented the deafferentation-induced decrease in phosphorylation of HSP27, strongly indicating that the mechanical load was a more essential stimulus in the regulation of HSP27.

Ikemoto et al. (17) reported that tail suspension of rats for 10 days or longer caused ubiquitination and degradation of MHC in gastrocnemius muscle, and the administration of a cysteine protease inhibitor to the suspended rats did not prevent MHC degradation, suggesting that unloading induced degradation of contractile proteins through a ubiquitin-dependent proteolytic pathway. In the present study, the increase of ubiquitinated MHC in the soleus muscle was also seen in the hindlimb-unloaded and deafferentated group (P > 0.05, Fig. 6), indicating that inhibition of mechanical load and/or neural activity promoted intramuscular protein degradation. The results also indicated that the deafferentation-related increase in ubiquitination was inhibited by the additional treatment with ablation of synergists, although the level of ubiquitinated MHC was not influenced by ablation of synergists alone. It is possible that fiber atrophy caused by inhibition of both mechanical load and neural activity was closely related to stimulation of proteolysis and also lowered protein synthesis, as suggested by decreased levels of phosphorylated S6 and HSP27.

In contrast, the compensatory hypertrophy of soleus muscle fibers caused by synergist ablation was accompanied with an increase in nucleolar number within a myonucleus and expression of phosphorylated S6 and HSP27. However, the level of ubiquitinated MHC remained unchanged. These results indicate that hypertrophy was induced mainly because of stimulated protein synthesis, although the number of myonuclei was stable.

In conclusion, hypertrophy of muscle and muscle fibers, associated with increased myonuclear domain size, nucleolar number in a single myonucleus, and phosphorylation levels of S6 and HSP27, was induced by the ablation of synergists, which increased the mechanical load in soleus muscle. In contrast, fiber atrophy, associated with decreased myonuclear number, domain size, and phosphorylation levels of S6 and HSP27 and increased myonuclear size and ubiquitination of MHC, was observed following hindlimb unloading, in which both mechanical load and neural activity were inhibited. Deafferentation-related atrophy of muscle, with inhibited EMG level, while the mechanical load was still maintained was also accompanied by decreased myonuclear domain size and phosphorylation of S6 and HSP27 and increased ubiquitination of MHC, although the magnitudes of the effects were minor compared with those in the hindlimb-unloaded rats. However, the myonuclear number was not affected by deafferentation, suggesting that the loss of myonuclei observed following hindlimb unloading may be closely associated with the inhibition of mechanical load in soleus muscle fibers. The deafferentation-induced degenerations of the abovementioned parameters were generally prevented by additional treatment with synergist ablation. Furthermore, data indicated that the number of nucleoli in a single myonucleus is increased by mechanical overloading irrespective of the EMG level. This suggests that load-dependent maintenance or upregulation of nucleolar number and/or phosphorylation of S6 and HSP27 plays an important role(s) in the regulation of muscle mass and also that such regulation is not necessarily associated with neural activity.

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