Long-term physical inactivity exacerbates hindlimb unloading-induced muscle atrophy in young rat soleus muscle

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Abstract

This study investigated the effects of long-term physical inactivity in adolescent on subsequent hindlimb unloading-induced muscle atrophy in rat soleus muscle. First, 3-wk-old male Wistar rats were assigned to an age-matched control (n = 6) or a physical inactivity (n = 8) group. Rats in the physical inactivity group were housed in narrow cages with approximately half the usual floor space for 8 wk to limit range of movement. Whole body energy consumption was measured, and the blood, organs, femoral bone, and hindlimb muscles were removed. We found that long-term physical inactivity did not affect the metabolic and physiological characteristics of growing rats. Then, fifty-six 3-wk-old male Wistar rats were assigned randomly into control (n = 28) and physical inactivity (n = 28) groups. After 8 wk, the rats in both groups underwent hindlimb unloading. The soleus muscles were removed before unloading (0 day), and 1, 3, and 7 days after unloading (n = 7 for each). Although the soleus muscle weight was significantly decreased after 7 days of hindlimb unloading in both groups, the decrease was drastic in the inactive group. A significant interaction between inactivity and unloading (P < 0.01) was observed according to the 4-hydroxynonenal-conjugated protein levels and the histone deacetylase 4 (HDAC4) and NF-κB protein levels. HDAC4 and NF-κB p65 protein levels in the physical inactivity group increased significantly 1 day after hindlimb unloading, along with the mRNA levels of their downstream targets myogenin and muscle RING finger protein 1 (MuRF1). Subsequent protein ubiquitination was upregulated by long-term physical inactivity (P < 0.05).

NEW & NOTEWORTHY Long-term physical inactivity exacerbates hindlimb unloading-induced disuse muscle atrophy in young rat soleus muscles, possibly mediated by oxidative stress-induced protein ubiquitination via HDAC4- and NF-κB p65-induced MuRF1 mRNA upregulation.

INTRODUCTION

In the past decade, physical inactivity (sedentary lifestyle) has been recognized as a risk factor for morbidity and mortality due to cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes worldwide (1, 2). Indeed, physical inactivity is the fourth leading cause of death worldwide, following high blood pressure, smoking, and high blood glucose (3, 4), making it one of the most important public health problems in the world.

Despite the awareness of the health risk of physical inactivity and the benefits of being physically active, physical activity levels remain inadequate worldwide because of an increase in sedentary behaviors (e.g., TV viewing, computer use), environmental factors (automation), and urbanization (5, 6).

Physical inactivity generally results in the so-called disuse syndrome (7, 8), which includes premature aging, obesity, cardiovascular vulnerability, musculoskeletal fragility, and depression (8). Considering that skeletal muscle is the most common and widely distributed muscle tissue in the body (comprising ~40% of the body’s total mass), the loss of skeletal muscle mass and strength can result in a drastic reduction in an individual’s quality of life and lead to an increased risk of development of insulin resistance and various chronic health conditions (9, 10). Indeed, even 2 wk of reduced daily step count by ~76% (1,400 steps) induced a significant decline in leg fat-free mass (~3.9%) and is accompanied with impairment of insulin sensitivity, systemic inflammatory makers, and myofibrillar protein synthesis in healthy elderly adults (11). Furthermore, McGlory et al. (12) demonstrated that 2 wk of reduced activity (~7,300 steps to 990 steps) lowered integrated muscle protein synthesis and insulin sensitivity in overweight and prediabetic older adults. Thus, the maintenance of skeletal muscle mass is necessary to...
maintain our health. Aging per se is associated with sarcopenia, which is the progressive loss of skeletal muscle mass and strength. Sarcopenia is associated with musculoskeletal frailty and impaired health span and quality of life in older individuals (13); however, this reproducible syndrome applies to young and middle-aged people as well as to elderly people.

A sedentary lifestyle during adolescence has a great impact on later life; thus, physical inactivity during adolescence poses a major problem for the maintenance of skeletal muscle and health (14, 15). During growth, physical inactivity can induce developmental disorders; however, in most cases, the abnormality is not easily discernible because organs and skeletal muscles continue to grow despite insufficient activity. Nevertheless, it may lead to significant interindividual variability in the growth process. Furthermore, no evidence has been established due to the difficulty of performing research in children and adolescents; therefore, animal experiments have been used to provide important insights into the investigation of the physical inactivity (16) -induced phenomena and mechanisms in growing children. Recent evidence revealed that 5 and 9 wk of physical inactivity, induced by living alone in a small cage with a floor space of 300 cm², caused endothelial dysfunction in healthy young mice (4 to 6 mo of age) (17). Takemura et al. (18) demonstrated that short-term (21 days) physical inactivity restricted by cage size (17.0 cm × 9.6 cm and 13.0 cm height vs. 31.5 cm × 9.6 cm and 13.0 cm height) decreased oxidative capacity and induced muscle atrophy in the muscles of 8-wk-old Wistar rats. Furthermore, Marmonti et al. (19) developed an immobilization model based on cage volume reduction (~80% reduction) that served as a physiological model for bed rest and suggested that severe cage volume reduction induces decreases in grip strength and soleus muscle mass and size.

However, whether long-term physical inactivity in childhood has impacts on the metabolic and physiological properties and exacerbates subsequent disuse-induced skeletal muscle atrophy is unclear. Therefore, we aimed to investigate the effects of long-term physical inactivity during adolescence on subsequent hindlimb unloading-induced muscle atrophy in rat soleus muscle. To address this issue, we examined the potential effects of 8 wk of physical inactivity during adolescence on the metabolic (whole body energy consumption and blood parameters) and physiological characteristics (organ weights and bone mechanical properties) and whether long-term physical inactivity exacerbates subsequent hindlimb-induced antigravity muscle atrophy in rat soleus muscle.

### MATERIALS AND METHODS

#### Experimental Animals and Design

This study was approved by the Juntendo University Animal Care Committee (Approval No. H26-09) and followed the principles for the care and use of laboratory animals set forth by the Physiological Society of Japan. Three-week-old male Wistar rats were housed in a climate-controlled room (temperature: 23°C ± 1°C, relative humidity: 55% ± 5%, 12:12-h light/dark cycle) and had free access to standard rat chow and water. The rats’ body weight and food intake were measured every week.

#### Experimental Procedure 1

**Physical inactivity model.**

The overview of experiments 1 and 2 are indicated in Fig. 1. After a 1-wk acclimation, the rats were assigned to an age-matched control (CT, *n* = 6) or a physical inactivity (IN, *n* = 8) group. Rats assigned to the IN group were housed in a small cage (2 rats per cage) with half of the usual floor space for 8 wk to limit their range of movement (Fig. 2A).
The floor space was gradually increased concurrently with the rats’ growth (198 cm² for 3-wk-old rats, ~272 cm² for 4- to 9-wk-old rats, ~408 cm² for 10- to 12-wk-old rats). The space provided for the CT group was similarly increased (198 cm² for 3-wk-old rats, 544 cm² for 4- to 9-wk-old rats, 880 cm² for 10- to 12-wk-old rats; 2 rats per cage).

**Measurement of spontaneous motor activity.**
To confirm the reduction in daily movement of the rats in the IN group, spontaneous motor activity was recorded using a commercial passive infrared sensor detection system (SUPERMEX; Muromachi Kikai Co., Ltd., Tokyo, Japan; Fig. 2B). The rats’ motor activity (4–12 wk old) was measured in a home cage (2 rats/cage) using the aforementioned system (20, Figure 2).
A sensor detects the radiated body heat of the animals and monitors motion in multiple zones of the cage through an array of Fresnel lenses placed above the cage that cover all three axes (X, Y, and Z). We measured the activity monitored by this system every 5 min for 24–48 h. Food and water were fully provided at the beginning of counting, and the rats were never disturbed in any way. The data were analyzed using CompACT AMS software v. 3 (Muromachi Kikai Co., Ltd.).

Whole body energy consumption analysis: indirect calorimetry measurements.

Indirect calorimetry, a noninvasive technique for measuring the mass of oxidation of carbohydrates (CHO) and fats through analysis of respiratory gas, was performed using the ARCO 2000 mass spectrometer (Arco System, Chiba, Japan) (22, 23), as described previously (24). Each rat was caged in an individual metabolic chamber maintained at 24°C between 7:00 a.m. and 10:00 a.m. (dark cycle) or between 10:30 a.m. and 13:30 p.m. (dark cycle). To mimic the usual housing condition, different sizes of chambers were used for each group (CT group: 32 × 19 × 14.5 cm³, 55.7% of the volume of the home cage; IN group: 22 × 10 × 13 cm³, 50.1% of the volume of the home cage; Fig. 3). Briefly, room air was pumped through an acrylic metabolic chamber at a rate of 1.5 L·min⁻¹ (1.2 L·min⁻¹ for the IN group), and the O₂ consumption (mL·kg⁻¹·min⁻¹) and CO₂ emission (mL·kg⁻¹·min⁻¹) were measured every 2.5 min for 3 h. The respiratory quotient (RQ), CHO oxidation (mg·kg⁻¹·min⁻¹), and fat oxidation (mg·kg⁻¹·min⁻¹) were calculated automatically from the O₂ consumption and CO₂ production using the Frayn equation (25). The data from the first 2 h were excluded and only the last 1 h was used (expressed as per min).

Blood and tissues sampling.

After 8 wk, the rats from both groups were anesthetized with isoflurane (3–5%) and pentobarbital sodium (60 mg·kg⁻¹), and blood samples were collected from both groups, then centrifuged at 3,000 rpm for 10 min to isolate the serum samples. The serum samples were rapidly frozen in liquid nitrogen and stored at −80°C until use. The blood parameters were measured by the Oriental Yeast Co., Ltd. (Tokyo, Japan). Serum corticosterone concentrations were estimated in duplicate using the Corticosterone EIA Kit (YK240; Yanaihara Institute Inc., Shizuoka, Japan) according to the manufacturer’s instructions.

The liver, spleen, kidney, adrenal, epididymal white adipose tissue, hindlimb muscles (soleus, plantaris, gastrocnemius, tibialis anterior, and extensor digitorum longus), and femoral bone were carefully removed and weighed.

Testing bone mechanical properties.

The bone mechanical strength of the right femur at midshaft was assessed with a three-point bending test, as previously described (26). Connective tissue was removed, and the bone was mounted on two support stands 10.6 mm apart. Then, the crosshead was applied to the metaphysis of the femur, which was compressed by the crosshead at a speed of 2.0 mm·min⁻¹ until fracture occurred. The maximum breaking force was divided by the femur weight and body weight.

Experimental Procedure 2

Hindlimb unloading and muscle sampling.

As with experiment 1, both groups of rats were assigned into the age-matched CT (n = 28) or IN (n = 28) groups. After 8 wk of inactivity, a group of rats (n = 21) were subjected to hindlimb unloading for 1, 3, or 7 days, as previously described (27). Briefly, a tail-cast suspension was applied to each rat, leaving the distal one-third of the tail free to allow proper thermoregulation. The tail cast was attached to a hook on the ceiling of the cage, and the height of the hook was adjusted so that the cast was inclined at an angle of ~35° in a head-down orientation. The rat was free to move around the cage on its front feet. The tails of the rats were checked daily for lesions or discoloration. Before unloading (0 day), and after 1, 3, or 7 days of hindlimb unloading (n = 7), the rats were anesthetized with isoflurane (3–5%) and pentobarbital sodium (60 mg·kg⁻¹); the soleus muscle was carefully removed and weighed. Muscle weight per body weight was expressed as relative muscle weight. The muscles were flash frozen in liquid nitrogen and stored at −80°C until analysis.

Muscle preparation.

Frozen soleus muscles were powdered, and a portion of the muscles (~30 mg) was homogenized in 10 volumes of ice-cold homogenization buffer (20 mM HEPES, pH 7.4, 4 mM EGTA, 0.1 mM EDTA, 10 mM MgCl₂, and 0.1% Triton X-100) containing complete EDTA-free and PhosSTOP protease inhibitor cocktails (both from Roche, Penzberg, Germany), as previously described (28). Homogenates were centrifuged at 900 × g for 5 min at 4°C, then the supernatants were centrifuged at 12,000 × g for 15 min at 4°C to collect pure cytosolic fractions. Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA).

Immunodetection.

Equal amounts of protein (10–20 μg) were loaded onto a precast 4%–15% Tris-glycine extended polyacrylamide gel (Bio-Rad, Copenhagen, Denmark) and electrophoretically separated at 150 V for 60 min. Separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) that was incubated in blocking buffer [5% nonfat dry milk in Tween-Tris-buffered saline (T-TBS: 40 mM Tris-HCl, 300 mM NaCl, and 0.1% Tween 20, pH 7.5)] for 60 min. The membrane was incubated with primary antibodies diluted by 3% bovine serum albumin in T-TBS for overnight: 4-hydroxynonenal (4-HNE, 1:1000; Abcam,
Cambridge, MA), monoubiquitinated and polyubiquitinated conjugates (Enzo Therapeutics, Farmingdale, NY), 20S proteasome subunit (1:2000; Abcam), histone deacetylase 4 (HDAC4) (1:2000, Cell Signaling Technology, Beverly, MA), NF-κB p65 (1:2000, Cell Signaling Technology), activating transcription factor 4 (ATF4, 1:2000, Cell Signaling Technology), forhead box subfamily O3a (Ser253) (FoxO3a, 1:2000, Cell Signaling Technology), and FoxO3a (1:2000; Millipore, Temecula, CA). This was followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10,000; Cell Signaling Technology) in dilution buffer (5% nonfat dry milk in T-TBS) for 1 h at room temperature (25°C–26°C). After several washes, protein bands were visualized using an enhanced chemiluminescence Prime reagent (GE Healthcare, Piscataway, NJ), and the signal was recorded using a ChemiDoc Touch imaging system (Bio-Rad). Signal intensity was analyzed using Image Lab version 5.2.1 (Bio-Rad). Immunodetection of total protein expressions or Ponceau red staining was performed for loading control.

Proteasome activity.
Proteasome activity was assessed using Proteasome 20S activity Assay Kit (Sigma, St. Louis, MO) following the manufacturer’s instruction. The chymotrypsin-like activity assay was conducted using skeletal muscle homogenates in a total volume of 100 μL in 96-well flat bottom black plates with clear bottoms. The proteasome LLVY-R110 substrate was added and incubated at 37°C for 1 h. The fluorescence was recorded as relative to proteasome 20S activity. Each sample was measured in duplicate.

Real-time polymerase chain reaction.
The total RNA was isolated as previously described (28). RNA was purified using an RNaseasy Mini kit (Qiagen, Valencia, CA.), and the concentration and purity (A260/280 and A260/230, respectively) were determined using QIAxpert (Qiagen). Reverse transcription was performed with 2 μg of total RNA using SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA). The mRNA levels of myogenin (Rn01490689_g1; Applied Biosystems, Foster City, CA), atrogin-1/muscle atrophy F-box (MAFbx) (Rn00591730_m1), muscle RING finger protein 1 (MuRF1) (Rn00590197_m1), and growth arrest and DNA damage-45 alpha (Gadd45 alpha; Rn01425130_g1) were quantified using a TaqMan gene expression assay (Applied Biosystems) and were normalized to 18S mRNA levels. The 2−ΔΔCt method was used for data analysis [cycle threshold (Ct) = Ct (gene of interest) – Ct (reference gene)], in which Ct indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Relative changes (ΔΔCt) in the expression level of target genes were calculated by subtracting the ΔCt of the CT group.

Statistical analysis.
Values were expressed as mean ± standard error of the mean. Statistical significance was evaluated by unpaired t test or two-way (Inactivity × Age for experiment 1 and Inactivity × Time for experiment 2) analysis of variance. Simple effects tests were performed when the interaction was significant. When significant main effects were found (without significant interaction), pairwise comparisons were performed where necessary using Sidak’s method. A P value <0.05 was considered statistically significant. All analyses were performed using Prism v. 6.0 (GraphPad Inc., La Jolla, CA).

RESULTS
Activity Counts
Figure 2 shows the typical pattern of the daily locomotor activity (Fig. 2C), daily activity count at 12 wk old (Fig. 2D), and weekly activity counts of the rats during the 8-wk experimental period (Fig. 2E). The IN group had a significant reduction in daily locomotor activity when compared with
Table 1. Data from whole body energy consumption analysis

| Variables                        | CT (n = 6) | IN (n = 8) |
|----------------------------------|------------|------------|
| VO₂, NmL·min⁻¹                   | 10.7 (0.6) | 9.5 (0.4)  |
| VCO₂, NmL·min⁻¹                  | 9.6 (0.4)  | 8.5 (0.3)  |
| VO₂, mL·kg⁻¹·min⁻¹               | 36.2 (2.0) | 33.7 (1.3) |
| VCO₂, mL·kg⁻¹·min⁻¹              | 32.7 (1.5) | 30.3 (1.0) |
| RQ, NmL·min⁻¹                    | 0.90 (0.01)| 0.90 (0.01)|
| Fat, mg·kg⁻¹·min⁻¹               | 1.88 (0.27)| 1.61 (0.24)|
| Carbohydrate, mg·kg⁻¹·min⁻¹      | 31.4 (0.6) | 29.6 (1.4) |
| Energy expenditure, mcL·min⁻¹    | 52.8 (2.7) | 46.6 (2.1) |

Values are means (SE). CT, control; IN, inactivity; RQ, respiratory quotient.

Table 2. Tissue weight, muscle weight, and bone mechanical properties

| Variables                        | CT (n = 6) | IN (n = 8) |
|----------------------------------|------------|------------|
| Liver, g                         | 9.5 (0.3)  | 9.1 (0.2)  |
| Spleen, mg                       | 712.9 (22.2)| 693.6 (12.0)|
| Kidney, mg                       | 966.8 (32.4)| 915.3 (16.3)|
| Adrenal mean, mg                 | 17.9 (1.5) | 18.3 (0.5) |
| Right adrenal, mg                | 17.8 (1.8) | 18.4 (0.4) |
| Left adrenal, mg                 | 18.1 (1.3) | 18.3 (0.8) |
| Epididymal white adipose tissue, g| 5.5 (0.2)  | 5.2 (0.2)  |
| Soleus muscle weight, mg         | 102.8 (3.7)| 94.8 (1.8) |
| Soleus muscle weight, mg·g·BW⁻¹  | 0.372 (0.007)| 0.342 (0.007)|
| Hindlimb muscles weight, mg      | 2,248.6 (50.1)| 2,260.3 (19.4)|
| Femoral length, cm               | 35.2 (0.3) | 34.7 (0.2) |
| Femoral weight, g                | 0.869 (0.029)| 0.854 (0.028)|
| Maximal breaking force, N         | 115.4 (4.2)| 120.0 (5.1) |
| Maximal breaking force, N·g·Fomur⁻¹| 133.4 (2.1)| 141.4 (6.7) |
| Maximal breaking force, N·g·BW⁻¹  | 0.393 (0.017)| 0.434 (0.030)|

Values are means (SE). BW, body weight; CT, control; IN, inactivity.

gastrocnemius muscles, tibialis anterior, and extensor digitorum longus) after 8 wk of physical inactivity.

Table 3. Blood parameters after 8 wk of physical inactivity

| Variables                        | CT (n = 6) | IN (n = 8) |
|----------------------------------|------------|------------|
| Triglyceride, mg·dL⁻¹            | 42.0 (5.6) | 33.3 (3.1) |
| Total-c, mg·dL⁻¹                 | 510.0 (1.4)| 526.2 (1.6)|
| HDL-c, mg·dL⁻¹                   | 173.0 (0.8)| 178.0 (0.6)|
| Calculated LDL-c, mg·dL⁻¹        | 25.3 (1.2) | 28.2 (1.3) |
| Glucose, mg·dL⁻¹                 | 146.7 (9.7)| 153.1 (2.1)|
| Creatine kinase, IU·L⁻¹          | 682.3 (128.6)| 585.8 (82.1)|
| BUN CRE⁻¹                        | 67.3 (2.4) | 81.2 (1.8) *|
| Corticosterone, ng·mL⁻¹          | 42.4 (1.2) | 41.6 (1.1) |

Values are means (SE). BUN, blood urea nitrogen; c, cholesterol; CRE, creatinine; CT, control; HDL, high density lipoprotein; IN, inactivity; LDL, low density lipoprotein. *P < 0.05.
4-HNE-Conjugated Proteins Levels

To establish if physical inactivity induced oxidative stress in the soleus muscle, we measured the 4-HNE-conjugated protein levels during hindlimb unloading (Fig. 6A). A significant interaction between inactivity and unloading was observed ($P = 0.0031$), and the protein levels 1 day after the onset of hindlimb unloading were significantly higher ($P < 0.0001$) in the IN group compared with the CT group (1.7- and 1.2-fold change from 0 day, respectively).

Ubiquitinated Protein Levels

The main effects of differences in inactivity ($P = 0.0356$) and unloading ($P = 0.0027$) were observed in ubiquitinated protein levels, which were higher in the IN group compared with the CT group 1 day after unloading (Fig. 6B).

Proteasome Activity and 20S Proteasome Subunit Protein Expression Levels

Figure 7 shows 20S proteasome activity and 20S proteasome subunit protein expression levels in the soleus muscle after 1 day, 3 days, and 7 days of hindlimb unloading. No differences between groups were observed in 20S proteasome activity and 20S proteasome subunit protein expression levels over the entire unloading period (Fig. 7).

HDAC4 and NF-κB Protein Expression Levels

A significant interaction between inactivity and unloading was observed in terms of the HDAC4 and NF-κB p65 protein expression levels ($P < 0.0001$ and $P = 0.0043$, respectively; Fig. 8). The HDAC4 and NF-κB p65 expression levels 1 day after the onset of hindlimb unloading were significantly higher ($P < 0.0001$ and $P = 0.0029$, respectively) in the IN group (3.9- and 2.0-fold change from day 0, respectively) compared with the CT group (1.4- and 1.2-fold change from day 0, respectively).

Activating Transcription Factor 4 Protein Expression and Forkhead Box Subfamily (Fox)O 3a (Ser253) Phosphorylation Levels

Figure 9 shows activating transcription factor (ATF) 4 protein expression levels and the ratio of phosphorylated FoxO3a (Ser253) in the soleus muscle during hindlimb unloading. No differences were observed between groups in ATF4 protein expression, and the ratio of phosphorylated FoxO3a over the entire unloading period (Fig. 9).

Muscle-Specific Atrophy-Related Gene Expressions

To assess potential alternations in the downstream targets of HDAC4 and NF-κB p65, mRNA expression levels of myo-
genin and E3 ligases atrogin-1 and MuRF1 as well as Gadd45α were determined after 1 day, 3 days, and 7 days of hindlimb unloading (Fig. 10). Although the atrogin-1/MAFbx mRNA level did not vary between the CT and IN groups, the myogenin, MuRF1, and Gadd45α mRNA levels were upregulated by hindlimb unloading in the IN group (\(P = 0.0042, \ P = 0.0370, \) and \(P = 0.0132, \) respectively). Moreover, the MuRF1 and Gadd45α mRNA expression levels 3 days after the onset of hindlimb unloading were significantly higher (\(P < 0.0001 \) and \(P = 0.0029, \) respectively) in the IN group (3.9- and 2.0-fold change from \(day \ 0, \) respectively) compared with the CT group (1.4- and 1.2-fold change from \(day \ 0, \) respectively).

**DISCUSSION**

In this study, we investigated the potential effects of 8 wk of physical inactivity during adolescence on the metabolic and physiological characteristics of growing rats, and whether long-term physical inactivity during adolescence exacerbates subsequent hindlimb-induced antigravity muscle atrophy in rat soleus muscle. We demonstrated that long-term physical inactivity did not affect the metabolic and physiological characteristics of growing rats; however, it exacerbated hindlimb unloading-induced muscle atrophy in the antigravity soleus muscle. Although the precise mechanisms are still unknown, these physical inactivity-related adverse effects on the soleus muscle during disuse may be mediated by oxidative stress-induced protein ubiquitination via HDAC4- and NF-κB p65, and their downstream target of MuRF1 mRNA upregulation.

Marmonti et al. (19), who developed an immobilization model based on the cage volume reduction (~80% reduction) as a physiological model for bed rest, suggested that severe cage volume reduction for 28 days induced decreases in grip strength and soleus muscle mass and size. In Figure 6.

![4-Hydroxynonenal-conjugated protein (A) and ubiquitinated protein (B) expression levels after 7 days of hindlimb unloading. Values are means ± SE; \(n = 7 \) per group. The results of two-way analysis of variance are displayed. \(* P < 0.05 \) vs. CT of each age. CT, control; IN, inactivity.](image1)

![20S proteasome activity (A) and 20S proteasome subunit protein expression level (B) after 7 days of hindlimb unloading. Values are means ± SE; \(n = 7 \) per group. The results of two-way analysis of variance are displayed. ns, not significant.](image2)
contrast, in this study (which limited physical activity to almost one-tenth of the normal condition), physical inactivity did not have a significant impact on whole body energy expenditure, organ weight, or muscle phenotype, and the bone mechanical properties due to cage volume restriction (~50% reduction) were not as severe as those of the previous study. Moreover, we found that the serum corticosterone level and adrenal weight, which are markers of stressors (29), did not differ between the groups; therefore, we have developed an adequate animal model for physical inactivity during adolescence without unnecessary restraint stress.

Importantly, we demonstrated that the disuse muscle atrophy in the antigravity soleus muscle was very drastic in inactive rats. Furthermore, we found that 8 wk of physical inactivity exacerbated hindlimb unloading-induced oxidative stress, as shown in Fig. 6. The level of 4-HNE protein, an oxidative specific marker of lipid peroxidation, contributed to skeletal muscle atrophy (30, 31). Increased oxidative stress is associated with upregulation of proteolytic markers such as protein ubiquitination (32). A previous study showed that protein ubiquitination in the soleus muscle increased after short-duration hindlimb unloading (~8 days) and was correlated with decreases in muscle weight (33), which suggested that the ubiquitination of proteins is a critical step in its degradation. In our study, although we could not find significant changes in 20S proteasome activity and 20S subunit expression levels, we observed higher levels of ubiquitinated protein in the soleus muscle in the inactive rats, which indicated an increased breakdown of muscle components as compared with the control rats during the early stage of unloading which resulted in a greater decrease in soleus muscle mass.

Interestingly, we also demonstrated in this study that 8 wk of physical inactivity induced greater HDAC4 expression levels after 1 day of unloading. Recently, the protein deacetylase HDAC4 has attracted attention as a central component of muscle atrophy, including denervation, immobilization and hindlimb unloading, which regulates E3 ligases mediated by myogenin. HDAC4 represses the expression of Dachshund 2 (Dach2), a negative regulator of myogenin, thereby activating the expression of E3 ligases, which participate in the proteolytic pathway and result in muscle atrophy (28, 34, 35). Indeed, we showed that physical inactivity had significant effects on the upregulation of myogenin and MuRF1 gene expression, and subsequently induced a high level of protein ubiquitination. Moreover, HDAC4 is an important regulator of Gadd45α for denervation-induced muscle atrophy (36). Gadd45α alters skeletal muscle gene expression and stimulates protein breakdown, reduces protein synthesis,
decreases the number of mitochondria, inhibits anabolic signaling, and, ultimately, causes muscle fiber atrophy (37). A previous study by Baehr et al. (38) demonstrated that Gadd45α mRNA expression was increased significantly after 3 and 7 days of unloading in the gastrocnemius muscle of adult rats. In line with these previous studies, we found that hindlimb unloading significantly affected Gadd45α mRNA expression, but the increase was more drastic with physical inactivity. Another important observation is that upregulation of NF-κB p65 expression was induced by physical inactivity in the soleus muscle. The members of the NF-κB family of transregulators are activated by a broad range of signals, including cytokines, mitogens, free radicals, and stress (39, 40). A previous study demonstrated that NF-κB is one of the most important transcription factors involved in muscle atrophy and that its activation can cause increased expression of proteasome subunits and ubiquitin ligase MuRF-1, and results in myofibrillar protein degradation (41). These serial data suggest that physical inactivity-induced HDAC4 and NF-κB p65 upregulation plays an important role in oxidative stress-induced protein ubiquitination and can drastically reduce soleus muscle weight in inactive rats, which may be mediated by downstream MuRF1 gene expression.

Notably, we observed that the effects of long-term physical inactivity occurred only in the soleus muscle at least after 7 days of hindlimb unloading. Regarding this point, a recent study by Nakamura et al. (42) demonstrated that exercise experience (24 m/min for 40 min/day, 5 days/wk for 8 wk) 8 wk before hindlimb unloading provided protective effects on the fast-twitch plantaris muscle than on slow-twitch soleus muscle. This study implies that increased muscle activity compared with normal habitation would contribute to the prevention of disuse stimuli; however, this muscle activity does not exhibit the protective effect of the physical activity. Thus, reduced physical activity, including a sedentary lifestyle and physical inactivity, during adolescence has more impact on slow-twitch soleus muscle since the fast-type muscles are less sensitive to disuse than the slow-type and postural muscles (43, 44). Nevertheless, a longer period of hindlimb unloading (≥ 2 wk) or different experimental conditions, such as using different atrophy models or different strains of rats/species, shows different adaptations to disuse. Therefore, further study is required to clarify the effects of long-term physical inactivity and following muscle disuse on the different types of muscles.

Our study has some limitations that should be addressed. First, although we could not observe any significant difference in the whole metabolic expenditure measurement, the condition we measured was not the same as that measured during inactivity because the rat remained in the metabolic chamber alone, and there were different sizes of cages between the groups. The rats were not able to communicate with each other in the cage; hence, we could not determine if the movement during the measurement was similar to that of the usual condition. Furthermore, we performed a short duration of measurement (3 h) to minimize the effects of the aforementioned unusual (in a metabolic chamber alone) condition; therefore, we should consider stress response to a new environment. Second, we did not directly assess the production of reactive oxygen species, the levels of oxidative stress,
stressed muscle by oxidative stress and HDAC4 and NF-κB p65 expressions are primary mechanisms that induce greater muscle atrophy in inactivating rats. Moreover, additional analyses of epigenetic modification and DNA methylation following physical inactivity would explain how long-term physical inactivity regulates upregulation of oxidative stress and HDAC4 and NF-κB p65 expression. Nevertheless, our data suggested that long-term physical inactivity exacerbated hindlimb unloading-induced muscle atrophy in the antigravity soleus muscle via oxidative stress-induced protein ubiquitination through HDAC4- and NF-κB p65 and their downstream target of MuRF1 mRNA upregulation without significant changes in the metabolic and physiological characteristics of growing rats. This study provides informative evidence that a sedentary lifestyle during adolescence has an adverse impact on the muscle phenotype during muscle wasting.

In conclusion, our data suggest that long-term physical inactivity exacerbates hindlimb unloading-induced muscle atrophy in young rat soleus muscle, which may be mediated by oxidative stress-induced protein ubiquitination via HDAC4- and NF-κB p65 and their downstream target of MuRF1 mRNA upregulation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

T.Y., R.K., S.M., and H.N. conceived and designed research; T.Y., T.T., and S-W.C. performed experiments; T.Y., T.N., and S-W.C. analyzed data; T.Y., T.N., S.M., and T.S. interpreted results of experiments; T.Y. prepared figures; T.Y. drafted manuscript; T.Y., T.N., T.T., S-W.C., R.K., S.M., T.S., and H.N. edited and revised manuscript; T.Y., T.N., T.T., S-W.C., R.K., S.M., T.S., and H.N. approved final version of manuscript.

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