Biochemical Adaptations in a Slow and a Fast Plantarflexor Muscle of Rats Housed in Small Cages

Ai Takemura; Roland R. Roy; V. Reggie Edgerton; Akihiko Ishihara

BACKGROUND: Chronic unloading and restricted activity are distinctly different processes, i.e., unloading completely removes the load on postural muscles, whereas restricted activity allows for loading of postural muscles. There are limited data available on the effects of restricted activity on skeletal muscles. Thus the effects of restricted activity on the properties of the slow soleus and fast plantaris muscles in rats were examined.

METHODS: Eight-week-old rats were housed for 21 d in normal-sized (control group) or in small-sized (restricted group) cages.

RESULTS: Decreased mRNA levels of peroxisome proliferator-activated receptor γ coactivator-1α (81 and 85% of control values) and reduced succinate dehydrogenase activity (85 and 88% of control values) were observed in the soleus and the plantaris muscles of the restricted group, respectively. Increased mRNA levels of forkhead box-containing protein O1 (128% of control values), decreased muscle weight (74% of control values), and reduced cross-sectional areas of type IIA (89% of control values) and type IIB (80% of control values) fibers were observed in the plantaris muscle of the restricted group.

DISCUSSION: Restricted activity decreased the mRNA levels of peroxisome proliferator-activated receptor γ coactivator-1α and increased the mRNA levels of forkhead box-containing protein O1, which are associated with reduced oxidative capacity and atrophy, respectively, in the muscles. The plantaris muscle was more affected by restricted activity than the soleus muscle, most likely reflecting a greater relative change in the normal activity pattern in the fast than slow plantarflexor muscle.

KEYWORDS: FOXO1, oxidative capacity, muscle fiber, PGC-1α, restricted activity.

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Skeletal muscles are routinely classified broadly into two types, i.e., slow and fast. Slow muscles such as the soleus are comprised predominantly of type I and type IIA fibers.[7] The slow muscles are active during low intensity and long duration activities, when performing functions against gravity, e.g., walking and maintaining posture.[26] In contrast, fast muscles such as the plantaris are comprised predominantly of type IIA and type IIB fibers.[7] The fast muscles are involved primarily during high intensity activities of short durations, when performing functions of strength and power are required.[26]

Skeletal muscles adapt to the level of neuromuscular activity (loading and activation) imposed on them. For example, a lack of load on skeletal muscles with hindlimb unloading[8,9,21] or exposure to microgravity by spaceflight[2,6,25] has a significant impact on the mechanical, biochemical, and morphological properties of the muscles. A chronic unloading causes atrophy in fibers of all types, especially type I fibers, a shift of fiber type from type I to type II, de novo synthesis of type IIx, reduced succinate dehydrogenase (SDH) activity, and decreased mRNA levels of heat shock proteins (HSPs) such as HSP27, HSP70, and HSP84.[6,8,9] A decrease in the mRNA levels of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and an increase in the mRNA levels of forkhead box-containing protein O1 (FOXO1) also have been observed after unloading.[21]

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In general, the effects are the greatest in the skeletal muscles predominantly comprised of slow fibers, such as the soleus.

The data on the effects of restricted activity on skeletal muscles are limited. Chronic unloading and restricted activity are distinctly different processes, i.e., unloading (e.g., hindlimb unloading and exposure to microgravity) completely removes the load on postural muscles, whereas restricted activity allows for loading of the postural muscles. Therefore, we hypothesized that muscle responses to chronic restricted activity would be different from those reported with chronic unloading.

The purpose of this study was to determine the effects of restricted activity imposed by housing the animals in small-sized cages on the properties of the slow soleus and fast plantaris muscles in rats. We hypothesized that the effects would be greater on the fast muscles due to the relatively greater reduction in the recruitment of fast neuromuscular pools compared to that of the more readily recruited slow neuromuscular pools during spontaneous cage activity.

METHODS

Animals

All experimental and animal care procedures were conducted in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Care and Experiment Committee of Kyoto University (Kyoto, Japan).

Eight-week-old male Wistar rats were divided into the control and restricted groups ($N = 6$ rats/group). Rats in the control group were housed in normal-sized cages (31.5 cm × 21.5 cm and 13.0 cm height) for 21 d, whereas those in the restricted group were housed in small-sized cages (17.0 cm × 9.6 cm and 13.0 cm height). We recorded the distance that the rats covered in their cages in each day during the 21 d of the experimental period by using an infrared video camera (DC-NCR13U, Medical Agent Inc., Kyoto, Japan) connected to a personal computer. All rats had access to a standard diet and water ad libitum. The room was maintained under a controlled 12 h light/dark cycle (light period from 08:00 to 20:00 h) at 22 ± 2°C with 45–55% relative humidity.

Procedures

At the end of the experimental period, the rats were anesthetized using sodium pentobarbital (35 $mg \cdot kg^{-1}$, i.p.). The soleus and plantaris muscles were excised bilaterally and any excess fat and connective tissues removed and then wet weighed. The rats then were killed by an overdose of sodium pentobarbital.

The left muscles were divided into two sections (distal and proximal) for measuring SDH activity and for analyzing mRNA. The distal part of the left muscle was immediately frozen and homogenized in five volumes of ice-cold 0.3 M phosphate buffer (pH 7.4) using a glass tissue homogenizer and then used for measuring SDH activity.[20,22] The components of the reaction mixture were as follows: 17 $mmol \cdot L^{-1}$ sodium succinate, 1 $mmol \cdot L^{-1}$ sodium cyanide, 0.4 $mol \cdot L^{-1}$ aluminum chloride, and 0.4 $mmol \cdot L^{-1}$ calcium chloride. The reduction in cytochrome *c* in this reaction mixture was analyzed using a spectrophotometer by observing an increase in the extinction at 550 nm. SDH activity was calculated from ferricytochrome *c* concentrations and protein contents.

Total RNA was extracted from the proximal part of the left muscle using TRIzol (Invitrogen, Carlsbad, CA). The muscles were treated with deoxyribonuclease I (Invitrogen). The first strand of cDNA was synthesized from 1.0 μg of total RNA using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). Gene expression was analyzed using real-time polymerase chain reaction (RT–PCR) performed on the LightCycler system DX400 (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq II (Takara Bio Inc.). The primer sets used in this study have been described previously.[21] The mRNA levels were normalized to those of the control group.

The right muscles were gently stretched to approximately their lengths in vivo, pinned on cork, and then rapidly frozen in isopentane cooled by dry ice and acetone. The muscles were mounted onto specimen chucks using Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Serial transverse sections (16 μm in thickness) were cut on a cryostat at -25°C. The sections were warmed to room temperature, air dried, and preincubated in acidic (pH 4.5) and alkaline (pH 10.4) conditions for the subsequent assessment of ATPase staining intensity. In each section, soleus muscle fibers were classified as type I (based on positive response to preincubation at pH 4.5 and negative response to preincubation at pH 10.4), type IIA (based on negative response to preincubation at pH 4.5 and positive response to preincubation at pH 10.4), and type IIC (based on positive response to preincubation at pH 4.5 and pH 10.4).[21–23] Plantaris muscle fibers were classified as type I (based on positive response to preincubation at pH 4.5), type IIA (based on negative response to preincubation at pH 4.5), and type IIB (based on intermediate response to preincubation at pH 4.5).[15,19,21] The fiber type composition and mean fiber type specific cross-sectional area were determined for approximately 200 fibers located in the central region of the muscle section. The cross-sectional area of the fibers was measured by tracing the outline of each fiber using a computer-assisted image-processing system (Neuroimaging System, Kyoto, Japan).

The sections were stained for 10 min to determine the SDH staining intensity of the fibers.[21–23] The SDH staining intensity was determined in the 200 aforementioned fibers using a computer-assisted image-processing system (Neuroimaging System, Kyoto, Japan). The sectional images were digitized as gray-scale images. Each pixel was quantified as 1 of 256 gray levels; a gray level of 0 was equivalent to 100% light transmission, whereas a gray level of 255 was equivalent to 0% light transmission. The mean optical density (OD) of all the pixels, which were converted to gray level values, within a fiber was determined using a photographic calibration tablet with 21 steps of gradient-density ranges and the corresponding diffused density values.

Statistical Analysis

The data are reported as mean ± SD of 6 rats. A repeated-measures ANOVA was used to evaluate the growth-dependent differences in the body weight between the control and restricted groups. When the differences were found to be significant by ANOVA, individual group comparisons were made using the Scheffé's post hoc test. Statistical significance was set at $P < 0.05$ or $P < 0.01$.

Student's *t*-tests were used to evaluate the differences in distance moved by rats in cages, food intake, muscle weight, mRNA levels, and muscle fiber properties between the control and restricted groups. Statistical significance was set at $P < 0.05$ or $P < 0.01$.

RESULTS

The distance moved by the restricted group (27 ± 9 cm · d^{-1}) was lower than that by the control group (5482 ± 1081 cm · d^{-1}) ($P > 0.01$).

There was a growth-dependent difference in the body weight between the control and restricted groups [$F_{(1,10)} = 10.689$, $P < 0.01$] (**Fig. 1**). The mean body weights between days 13 and 17 were lower in the restricted group than in the control group ($P < 0.01$, $P < 0.05$, and $P < 0.05$ for days 13, 15, and 17, respectively). There was, however, no difference in the mean body weight at the end of the experimental period between the control and restricted groups ($P = 0.0507$).

There was no difference in the mean daily food intake during the 21 d of the experimental period between the control (15.8 ± 0.7 g · d^{-1}) and restricted (15.4 ± 0.7 g · d^{-1}) groups ($P = 0.3008$).

There was no difference in the mean soleus muscle weight between the control and restricted groups ($P = 0.1095$) (**Fig. 2A**). In contrast, the mean plantaris muscle weight was lower in the restricted group than in the control group ($P < 0.01$).

The mean SDH activity in both the soleus and plantaris muscles was lower in the restricted group than in the control group ($P < 0.05$ for the soleus and plantaris muscles) (**Fig. 2B**).

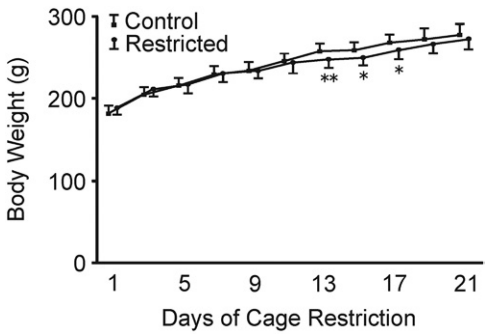

Fig. 1. Line graphs comparing the body weights of the control and restricted groups during the 21 d of the experimental period. Data are presented as the mean and the error bar (SD) for 6 rats. Note that the data points for the two groups are offset at each time point for clarity. *$P < 0.05$ and **$P < 0.01$ compared with the control group at each time point.

The mean mRNA levels of PGC-1α were lower in both the soleus and plantaris muscles in the restricted group than in the control group ($P < 0.05$ for the soleus and plantaris muscles) (**Fig. 3A**). There was no difference in the mean mRNA levels of FOXO1 in the soleus muscle between the control and restricted groups ($P = 0.1048$) (**Fig. 3B**). In contrast, the mean mRNA levels of FOXO1 in the plantaris muscle were higher in the restricted group than in the control group ($P < 0.05$).

The soleus muscles of both the control and restricted groups were comprised of three types of fibers, i.e., type I, type IIA, and type IIC fibers (**Fig. 4A-F**). There were no differences in the mean fiber type composition ($P = 0.3897$, $P = 0.1653$, and $P = 0.2497$ for type I, type IIA, and type IIC fibers, respectively) (**Fig. 5A**) or the mean cross-sectional area of each fiber type ($P = 0.1714$, $P = 0.1995$, and $P = 0.6725$ for type I, type IIA, and type IIC fibers, respectively) (**Fig. 5B**) between the control and restricted groups. The mean SDH staining intensity of type I and type IIA fibers was lower in the restricted group than in the control group ($P < 0.01$ for type I and type IIA fibers) (**Fig. 5C**). There was no difference in the mean SDH staining intensity of type IIC fibers between the control and restricted groups ($P = 0.6644$).

The plantaris muscles of both the control and restricted groups were comprised of three types of fibers, i.e., type I, type IIA, and type IIB fibers (**Fig. 4G-J**). There were no differences in the mean fiber type composition between the control and restricted groups ($P = 0.3081$, $P = 0.4028$, and $P = 0.8415$ for type I, type IIA, and type IIB fibers, respectively) (**Fig. 5D**). There were no differences in the mean cross-sectional area of type I fibers between the control and restricted groups ($P = 0.6113$) (**Fig. 5E**). The mean cross-sectional areas of the type IIA and type IIB fibers were smaller in the restricted group than in the control group ($P < 0.05$ and $P < 0.01$ for type IIA and type IIB fibers, respectively). The mean SDH staining intensity of each fiber type was lower in the restricted group than in the control group. ($P < 0.01$ for type I, type IIA, and type IIB fibers) (**Fig. 5F**).

DISCUSSION

Our model using restricted activity by housing animals in small-sized cages is different from the hindlimb unloading and exposure to microgravity models. Hindlimb unloading and exposure to microgravity completely remove the load on the postural muscles, whereas restricted activity allows for loading of the postural muscles. Thus, this study can be considered as a study of the effects of sedentary behavior.

The mean distance moved by the rats housed in small-sized cages was 0.5% of that moved by rats housed in normal-sized cages. The rats in both small- and normal-sized cages ate, drank water, and groomed during both their active (dark from 20:00-08:00) and nonactive (light from 08:00-20:00) periods. The rats in the small-sized cages had little space to move around and remained quite still even during the active period. In contrast, the rats housed in normal-sized cages moved around slowly in

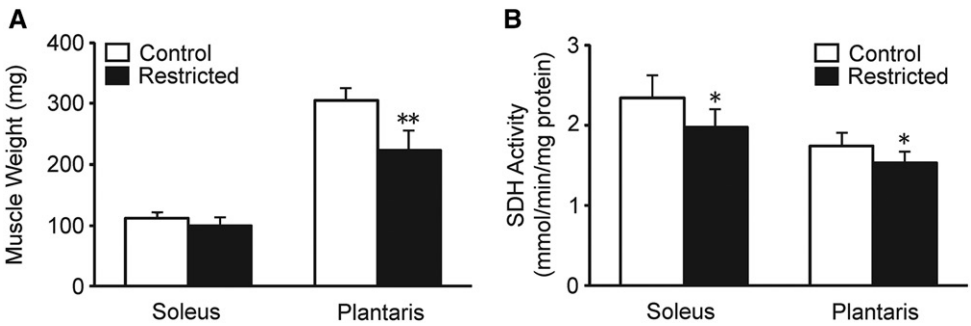

Fig. 2. A) Weights and B) succinate dehydrogenase (SDH) activity of the soleus and plantaris muscles in the control and restricted groups. Data are presented as the mean and the error bar (SD) for 6 rats. *$P < 0.05$ and **$P < 0.01$ compared with the control group.

the cages, especially during the active period. The shortest and longest distances covered by the rats in the normal-sized cages were 3073 cm · d^{-1} and 8341 cm · d^{-1}, respectively. These distances are 1.8% of those (3000 m · d^{-1}) covered by the age-matched rats that ran voluntarily in no-load wheels attached to the cages for 21 d in our previous study.[10] This relatively high level of activity resulted in a greater mass and higher oxidative capacity of the hindlimb muscles,[10,29] indicating that housing conditions such as the area and activity environment in the cages strongly affect skeletal muscle properties.

In this study, we determined the effects of restricted activity induced by housing in small-sized cages on the properties of the predominantly slow soleus muscle and the predominantly fast plantaris muscle in rats. The weights of the soleus and plantaris muscles were ~10% and 27% lower, respectively, in rats housed in small-sized cages than in those housed in normal-sized cages. Consistent with this observation, the mean cross-sectional areas of the fiber of some types in the plantaris muscle (Fig. 5E), but not in the soleus muscle (Fig. 5B), were smaller in the restricted group than in the control group. Thus, restricted activity had a greater effect on the fast muscle than on the slow muscle. These results were different from previous findings on the effects of hindlimb unloading[8,9,21] or exposure to microgravity,[2,3,25] which showed a greater atrophy of slow than fast muscles. The slow soleus muscle is recruited heavily during low weight-bearing activities such as during standing and walking, whereas the fast medial gastrocnemius and plantaris muscles are recruited during higher intensity activities such as during fast walking, running, and jumping.[26] Unloading (e.g., hindlimb unloading and exposure to microgravity) completely removes the load on postural muscles, whereas restricted activity allows for loading of the postural muscles. Thus, it is likely that the relative activity level of the soleus muscle was maintained closer to normal in the small-sized cages than that of the plantaris muscle, in that the rats were able to stand quadrupedally in the small-sized cages but could not perform more intense sustained movements.

The FOXO1 protein belongs to the forkhead family of transcription factors that are characterized by a distinct forkhead domain.[4,5,24] FOXO1 regulates myogenic differentiation and growth, muscle atrophy, and glycemic properties.[11–13] It also promotes the expression of atrogin-1, a muscle-specific ubiquitin ligase, which, along with muscle RING-finger protein-1 (MuRF1), plays a role in controlling muscle atrophy.[28] Mice overexpressing FOXO1 show a downregulation in slow muscle genes and a decrease in muscle mass, suggesting that FOXO1 determines myogenic lineage specification.[11] Consistent with these observations, it also has been shown that FOXO1 ablation in skeletal muscles results in a type shift to MyoD-containing fast fibers and alters the fiber type composition at the expense of myogenin-containing slow fibers.[12]

The increase in the mRNA levels of FOXO1 in the plantaris muscle of the restricted group (Fig. 3B) is consistent with the changes observed in the muscle and its fibers, i.e., a decrease in the muscle weight (Fig. 2A) and cross-sectional area of the type IIA and type IIB fibers (Fig. 5E). FOXO1 overexpression is induced during fasting as a means of maintaining energy homeostasis through utilization of lipids rather than carbohydrates as the energy source in the skeletal muscles.[1] This increase in the mRNA levels of FOXO1 in the skeletal muscle during starvation has been linked to decreased protein synthesis and increased protein degradation, leading to muscle loss and atrophy.[11,27,30] In addition, hindlimb unloading-induced muscle and fiber (all types) atrophy in the soleus and plantaris muscles with increased mRNA levels of FOXO1 has been reported.[21] In this study, atrophy (Fig. 2A) and a decrease in the cross-sectional area of type IIA and type IIB fibers (Fig. 5E) were observed in the plantaris muscle. Thus, it appears that one contributor to the observed atrophy of the plantaris muscle and its fibers may be the increase in the mRNA levels of FOXO1.

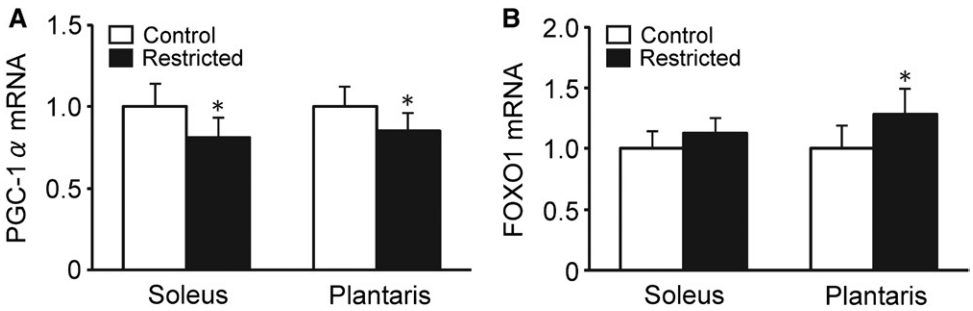

Fig. 3. A) mRNA levels of the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and B) forkhead box-containing protein O1 (FOXO1) in the soleus and plantaris muscles of the control and restricted groups. Data are presented as the mean and the error bar (SD) for 6 rats. *$P < 0.05$ compared with the control group.

PGC-1α is a member of a family of transcription coactivators that plays a central role in the regulation of glucose/fatty acid metabolism, mitochondrial

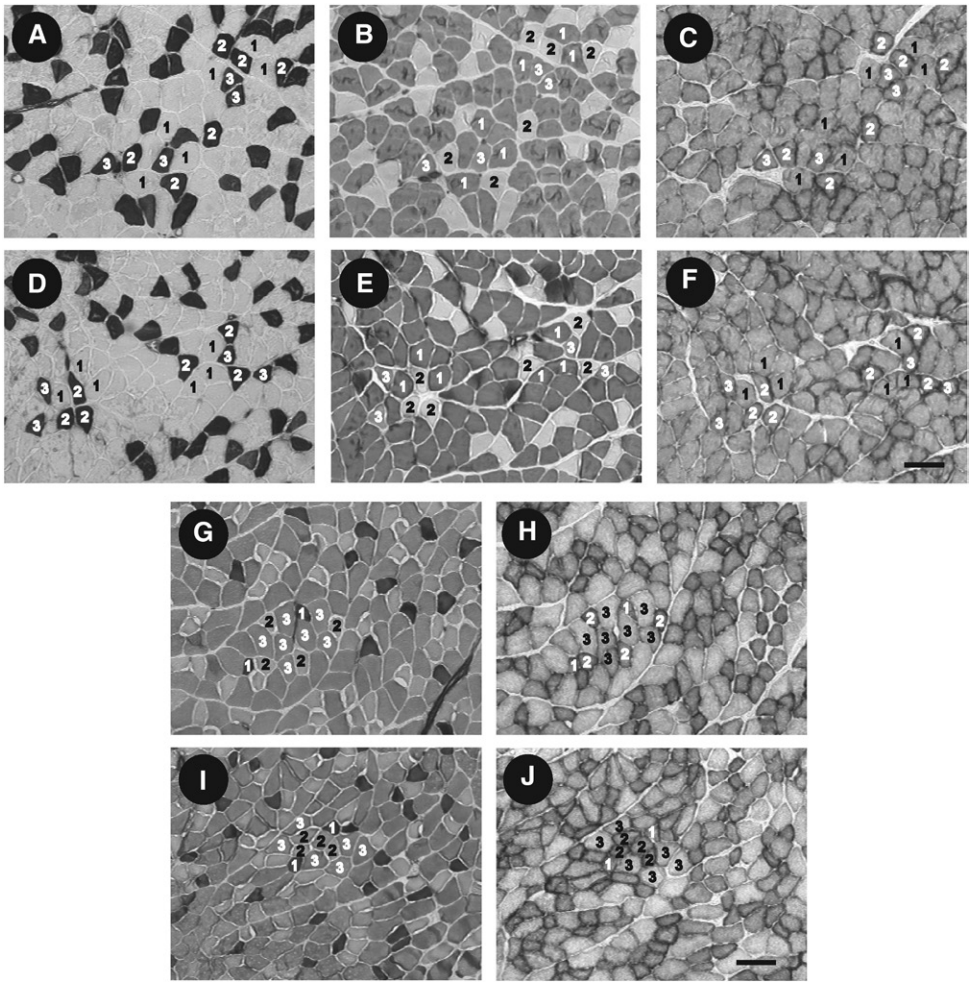

Fig. 4. Serial transverse sections of the soleus muscles from the rats in the control (A-C) and restricted (D-F) groups and of the plantaris muscles from the rats in the control (G, H) and restricted (I, J) groups. The sections were stained for adenosine triphosphatase activity after preincubation at pH 10.4 (A, D) and pH 4.5 (B, E, G, I), as well as for succinate dehydrogenase activity (C, F, H, J). In panels A-F: 1 = type I; 2 = type IIA; 3 = type IIC. In panels G-J: 1 = type I; 2 = type IIA; 3 = type IIB. Scale bar in F and J = 100 μm.

synthesis, vascularization, proteolysis, and apoptosis.[14,31] PGC-1α is expressed at high levels in tissues where mitochondria are abundant and oxidative metabolism is active in brown adipose tissues, heart, and skeletal muscles. In skeletal muscle, PGC-1α accelerates mitochondrial synthesis and promotes the remodeling of fibers. For example, transgenic mice with an increased expression of PGC-1α in the skeletal muscle show a decrease in ATP, an increase in mitochondria, and a shift of fiber types, i.e., a relative increase in metabolically more oxidative and less glycolytic fibers.[16,18] In addition, at 25 wk of age, these transgenic mice show fiber atrophy with depletion of ATP in predominantly fast muscles such as the gastrocnemius and quadriceps muscles.[17]

In this study, restricted activity resulted in a decrease in the oxidative capacity of both the soleus and the plantaris muscles, as reflected by a decrease in the SDH staining intensity in most of the fiber types (Fig. 5C, F). Nagatomo et al.[21] reported a decrease in the oxidative enzyme activity, SDH staining intensity of type IIA and type IIC fibers, and the mRNA levels of PGC-1α in the soleus muscle after hindlimb unloading. In contrast, the oxidative enzyme activity, SDH staining intensity of all types of fibers, and the mRNA levels of PGC-1α in the plantaris muscle did not change after hindlimb unloading,[21] indicating that the reduced muscle oxidative capacity was related to the decreased mRNA levels of PGC-1α. Therefore, we conclude that the reduced oxidative capacity in both the soleus and the plantaris muscles in the restricted group is associated with the observed decrease in the mRNA levels of PGC-1α.

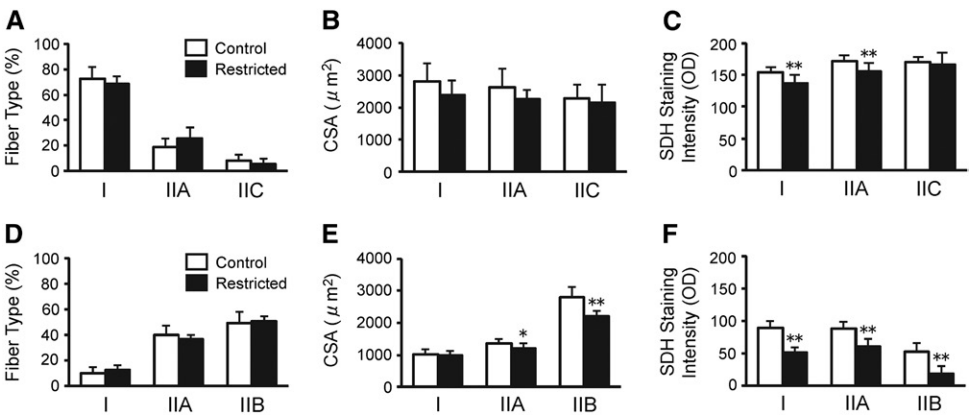

Fig. 5. Fiber type compositions (A, D), cross-sectional areas (B, E), and succinate dehydrogenase (SDH) staining intensity (C, F) for each type in the soleus (A-C) and plantaris (D-F) muscles of the control and restricted groups. CSA, cross-sectional area; OD, optical density. Data are presented as the mean and the error bar (SD) for 6 rats. *$P < 0.05$ and **$P < 0.01$ compared with the control group.

In summary, restricted activity resulted in a decrease in the mRNA levels of PGC-1α and an increase in the mRNA levels of FOXO1, which are associated with decreased oxidative capacity and atrophy of skeletal muscles, respectively. Restricted activity affected the fast plantaris muscle to a greater extent than the slow soleus muscle, most likely

reflecting a greater relative change in the normal activity pattern in the fast muscle than in the slow muscle. These results indicate that housing conditions such as the area and activity environment in the cages strongly influence skeletal muscle properties, such as the oxidative capacity and mass. The results indicate that this model of restricted activity may provide insight into what occurs in humans during a prolonged stay in a small space, e.g., in a submarine that sinks into the deep sea, in a search vehicle that explores the bottom of the earth, and in a rover for the observation of regions with severe environments, hazards, and/or disasters.

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