Satellite cell regulation of muscle mass is altered at old age

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Hindlimb suspension (HS) of rodents has been utilized to mimic bed rest and to investigate the underlying mechanisms associated with the loss of muscle mass due to inactivity (35). HS is associated with a decrease in muscle mass mainly of postural muscles such as the soleus; other hindlimb muscles, such as the extensor digitorum longus, atrophy to a much lesser extent or not at all (for review, Ref. 58). The effects of HS on muscles of old animals are controversial. Some studies report that soleus muscles atrophy to a similar degree in young and old rats (56, 60, 61), whereas others suggest that soleus muscles from young animals atrophy more than those from old animals (12, 55). We and others have shown that as little as 1 h of regular cage activity each day decreased the atrophy in young animals (7, 21, 27, 50, 67). Although mechanisms underlying maintenance of muscle mass during disuse in old animals has hardly been explored, restoration of muscle mass after a period of disuse has been studied. It was shown that muscles from old animals did not recover muscle mass lost during immobilization imposed by casting or external fixation, even after long periods of recovery, whereas those from young animals did (15, 68). Also, recovery of muscle mass lost due to starvation is impaired in old compared with young rats (46). These studies imply that the underlying mechanisms regulating muscle size likely change with aging.

Changes in skeletal muscle size are generally accompanied by alterations in the number of myofiber nuclei, such that a more or less constant myonuclear domain (amount of cytoplasm per nucleus) is maintained (4). Only a few studies have investigated the regulation of myonuclear domain with aging. One study showed that myonuclear domain remained unchanged with aging (63), whereas another showed that it decreased (38). Atrophy is associated with a loss of nuclei likely due to apoptotic processes (2, 28), and skeletal muscle hypertrophy is correlated with an increase in myofiber nuclei (3, 13, 41). Satellite cells are thought to serve as the source of new myofiber nuclei. Satellite cells are undifferentiated myogenic stem cells located between the muscle fiber plasma membrane and the basement membrane (40) and have been shown to be important during normal muscle growth, regeneration, hypertrophy, and recovery after atrophy (8, 32, 43, 53). When stimulated, satellite cells become activated and undergo cell division, after which they fuse into the muscle fiber (for

SKELETAL MUSCLE MASS GRADUALLY declines with advancing age. By 60–70 yr of age, muscle mass in human beings has decreased by 25–30%, and this decrease is due to a loss of fibers as well as a decrease in total cross-sectional area of the remaining fibers (11, 30, 36). The loss of muscle mass, and concomitant muscle strength with aging (11), has been termed sarcopenia (reviewed in Ref. 25). Muscle weakness has been found to be a common feature in the elderly who suffer falls (62) and is associated with increased mortality (42). Moreover, the elderly often experience periods of inactivity due to bed rest, and this results in a rapid and significant loss of skeletal muscle mass, particularly in postural muscles such as the soleus (33). It is unknown at this time whether old individuals recover from disuse due to bed rest to the same extent as younger individuals or whether the amount of therapy needed to prevent the loss of muscle mass during disuse is similar for different ages.

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review, Ref. 8). It is likely that satellite cell proliferation is required for hypertrophy to occur, because γ-irradiation, which kills dividing cells, prevented hypertrophy (49, 53) and recovery from lengthening contraction-induced muscle injury (51). Recently, Mitchell and Pavlath (43) showed that satellite cells are also required for complete recovery from atrophy induced by HS. Therefore, the ability of muscles to recover from atrophy is dependent on the properties of satellite cells present within the muscle. In older individuals and animals, it has been shown that satellite cells exhibit distinct functional properties. Satellite cells from old rats displayed a longer lag period before entering the cell cycle, compared with cells from young rats (24, 34), and the proliferation potential of muscle stem cells decreases as a function of age (54). In humans, skeletal muscle stem cells showed a steady decline in replicative capacity and in muscle-specific gene expression in vitro as the age of the donor increased (48, 52, 65). Furthermore, the number of satellite cells present in muscle also decreases with age (1, 10, 31, 52), whereas their ability to proliferate and differentiate seems to be retained (10, 52). However, satellite cells isolated from old mice showed an increased adipogenic potential compared with cells from younger mice (57), and, recently, it was shown that the impaired regenerative potential of old muscle was due in part to diminished activation of Notch signaling (18). These studies indicate that the functional properties of satellite cells are altered at old age and could, therefore, influence the control of muscle size. Satellite cell function also changes after an atrophy-inducing event such as HS. In growing rats, satellite cell proliferative activity was severely reduced after HS (20), and, in adult mice, it has been shown that atrophy decreased survival and myogenicity in muscle cells (44).

This study was designed to investigate whether maintenance of muscle mass in the midst of an atrophy-inducing condition is impaired with aging and whether the decrease in satellite cell function with aging is a contributing factor. We hypothesize that aging is associated with altered satellite cell function affecting myonuclear domain.

MATERIALS AND METHODS

Animals and Experimental Procedures

All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals. Male Fischer 344 X Brown Norway rats, aged 6, 20, and 32 mo, were purchased from the National Institute on Aging. This strain of rat was chosen because it has increased longevity and decreased cumulative lesion incidence compared with other strains, and, therefore, aging aspects can be studied in the relative absence of disease (37). The different ages were chosen to reflect a mature rat (6 mo), a middle-aged rat (20 mo), and an aged rat at ~50% mortality (32 mo). Rats of the three ages were divided into 4 groups (n = 6 per group): 1) control, nonsuspended; 2) hindlimb suspended for 7 days (HS7); 3) hindlimb suspended for 14 days (HS14); and 4) hindlimb suspended with intermittent reloading for 14 days. Rats were allowed free access to food and water and were housed in a 12:12-h light-dark cycle. Rats in the hindlimb-suspended and reloading groups were suspended as previously described (27). Briefly, a tail device containing a hook was attached with gauze and cymocrylate glue while the animals were anesthetized with pentobarbital sodium (50 mg/kg). During the anesthesia, a continuous release pellet containing 5'-bromo-2'-deoxyuridine (BrdU) (Innovative Research America, Sarasota, FL), constructed to give a dose of 0.022 mg BrdU·g body wt−1·day−1, was implanted subcutaneously in the subscapular region (14). After the animals regained consciousness, the tail device was connected via a thin cable to a pulley sliding on a vertically adjustable stainless steel bar running longitudinally above a high-sided cage with standard floor dimensions. The system was designed in such a way that the rats could not rest their hindlimbs against any side of the cage. Animals in the HS14 with intermittent reloading group were allowed to move around their cage freely by unhooking the suspension device for 1 h at the same time each day during the first hour of the dark cycle. During the intermittent reloading, the animals were observed constantly, and animals of the different ages did not differ in their observed cage activity during this 1 h of reloading, which consisted mainly of grooming and exploring the cage. At the end of the experimental time period, rats were killed by an overdose of pentobarbital sodium, and the soleus and plantaris muscles were dissected, weighed, and frozen. Muscles from one leg were frozen in liquid nitrogen and stored at −80°C for RNA analysis, and muscles from the other leg were embedded in freezing medium, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for immunohistochemical analysis. Muscle weights are expressed as absolute muscle weights and as the ratio of muscle weight to body weight (mW/bW). Because there are differences in body weights between the ages and between experimental groups, it is useful to show the relative muscle weights as an indicator of sarcopenia.

RNA Isolation and Northern Blot Analysis

RNA isolation and detection were performed as described previously (26, 27). Briefly, total RNA was isolated from muscles by using the guanidinium thiocyanate-phenol-chloroform extraction method, as described by Chomczynski and Sacchi (16). Ten micrograms of total RNA were electrophoresed through 1% agarose/2% formaldehyde HEPEs/EDTA-buffered gels and separated for 2–3 h at 70 V. RNA was then transferred to a nylon membrane (Zeta-Probe, BioRad, Richmond, CA) by using a blotting unit (BIOS, New Haven, CT) and ultraviolet cross-linked by using a Stratalinkr (Stratagene, La Jolla, CA). Membranes were sequentially hybridized with the following cDNA probes: MyoD, myogenin, and 18S rRNA. All probes have been described previously (26, 27). Labeling of the probes was performed by using the random prime method, according to the manufacturer’s recommendations (Decaprime II kit, Ambion, Austin, TX). Hybridization was performed according to Church and Gilbert (17). Briefly, filters were hybridized overnight in rotating flasks in a hybridization oven (Robbins Scientific, Sunnyvale, CA) at 65°C in buffer containing 0.5% crystalline grade bovine serum albumin (Calbiochem, San Diego, CA), 1 mM EDTA, 0.5 M NaPO₄, pH 7.2, and 3% SDS. Filters were washed sequentially in wash solution A (1 mM EDTA, 40 mM NaPO₄, pH 7.2, 5% SDS), wash solution B (1 mM EDTA, 40 mM NaPO₄, pH 7.2, 1% SDS), and in postwash (1.6× SSC: 5 mM Tris, pH 8.0, 1 mM EDTA, 0.1% SDS) at 65°C for 40 min each. Filters were exposed to a phosphor screen, scanned by using a StormScan (Molecular Dynamics, Sunnyvale, CA), and density analysis of the bands was performed by using the ImageQuant software (Molecular Dynamics). Density of bands for MyoD and myogenin was normalized to density of the bands for 18S rRNA and expressed as arbitrary density units.

Control samples of the three different ages were run on one gel, and age comparisons were made on control samples only. Because not all of the samples could be run on one single gel, differences between the groups within one age group were made on a separate set of filters, and, therefore, no age comparisons could be made between experimental groups, except for controls. However, to display the data within one data set, all values within one age group were normalized to the control values of that particular age group (Table 1).
Table 1. Myofiber cross-sectional area of soleus muscles after hindlimb suspension with and without intermittent reloading

<table>
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<tr>
<th></th>
<th>6 mo</th>
<th>20 mo</th>
<th>32 mo</th>
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<tr>
<td>Control</td>
<td>2,976.8 ± 137.9</td>
<td>2,990.3 ± 225.6</td>
<td>2,184.4 ± 104.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>HS7</td>
<td>2,235.4 ± 150.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2,066.5 ± 104.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1,693.4 ± 228.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HS14</td>
<td>1,128.0 ± 61.9&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1,405.0 ± 48.6&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>926.2 ± 94.8&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>HSIR14</td>
<td>1,480.1 ± 47.5&lt;sup&gt;se&lt;/sup&gt;</td>
<td>1,850.9 ± 106.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1,231.6 ± 80.3&lt;sup&gt;e&lt;/sup&gt;</td>
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Values are means ± SE in μm<sup>2</sup>. HS7, hindlimb suspended for 7 days; HS14, hindlimb suspended for 14 days; HSIR, hindlimb suspended for 14 days and intermittently reloaded. Significantly different from *6 mo, †20 mo, ‡control, §HS7, and ¶HS14: P < 0.05.

Immunohistochemistry

Cross sections of soleus muscles were cut on a cryostat (8 μm), air dried, and stored at −20°C. To determine myofiber nuclear number, the sections were double stained for dystrophin and Hoechst, to identify nuclei within the sarcolemma (28, 29). A dystrophin antibody (mouse anti-human dystrophin, NCL-DYS2, Vector, Burlingame, CA) was applied at a 1:4 dilution, followed by a rat anti-mouse IgG1 alkaline phosphatase-conjugated secondary antibody (Pharmigen, San Diego, CA). The alkaline phosphatase substrate kit (Vector) was used for visualization. Sections were subsequently fixed in 2% paraformaldehyde, and Hoechst 33258 nuclear dye (Molecular Probes, Eugene, OR) was applied at 1.2 ng/ml for 30 min. Sections were viewed with a fluorescent microscope (Nikon, Eclipse E600. Melville, NY) by using an ultraviolet filter package, and images were captured by using imaging software (MetaView, Universal Imaging, West Chester, PA). Nuclei within the dystrophin-positive sarcolemma were counted in 250–300 fibers, and the nuclear number was expressed per fiber. Nuclei that did not touch the sarcolemmal dystrophin stain, but were inside the fiber, were counted as centrally located nuclei. Myofiber cross-sectional area (MCSA) was determined by analyzing a region of 150 fibers with the use of Metaview software, as described (26).

To identify nuclei that had undergone division, a BrdU/dystrophin double-staining protocol was performed as described previously (28, 29). To detect BrdU and dystrophin on the same section, muscle sections were permeabilized with 0.1% Igepal. BrdU antibody was applied at a concentration of 6–8 ng/μl and incubated for 1 h at room temperature. After washing, a secondary rat anti-mouse IgG1 biotin-conjugated antibody (Zymed, San Francisco, CA) was applied at 1:100 dilution for 1 h at room temperature. Streptavidin-peroxidase was applied followed by diamobenzidine peroxidase substrate (Vector). Five areas of 80–120 fibers from different sites in the muscle were counted for BrdU positivity, and numbers were expressed per 100 fibers. BrdU-positive nuclei inside the dystrophin-stained sarcolemma were counted, as well as BrdU-positive nuclei outside the sarcolemma.

Statistics

To test for statistically significant differences, two-way analysis of variance was used, except for the RNA samples, where one-way analysis of variance was applied. In the case of significant differences, the Tukey multiple-comparisons test was applied. Statistical significance was assumed at P < 0.05.

RESULTS

Skeletal Muscle Atrophy Is Not Attenuated With Intermittent Reloading at Old Age

**Effect of age.** Absolute muscle weights of soleus (Fig. 1A) and plantaris (Fig. 2A) did not change in control rats from 6 to 20 mo versus 6 mo old. Hindlimb suspension at 20 mo resulted in atrophy of soleus and plantaris muscles, but intermittent reloading ameliorated the atrophy.

![Fig. 1. Intermittent reloading does not attenuate muscle atrophy in old soleus muscle. Muscle weights (A) and muscle weight-to-body weight ratios (B) are shown for soleus muscles from control (solid bars), hindlimb suspended for 7 days (HS7; open bars), hindlimb suspended for 14 days (HS14; shaded bars), and hindlimb suspended and intermittently reloaded for 1 h each day for 14 days (HSIR14; shaded bars). Values are means ± SE. Significantly different from *6 mo, †control, §HS7, ¶HS14, and ††20 mo (P < 0.05).](www.jap.org)
20 mo of age, but decreased from 20 to 32 mo of age. Relative muscle weight (mw/bw), however, decreased with advancing age in both muscles of control rats from 6 to 20 and from 20 to 32 mo (Fig. 1B and 2B), confirming that sarcopenia is evident in these rats. MCSA was determined on soleus muscles to ensure that the loss of muscle mass reflected a decrease in myofiber size. MCSA measurements confirmed the results from the muscle weights; i.e., there is a loss of MCSA in soleus muscles at old age (32 mo), but no difference between 6 and 20 mo (Table 1).

**Effect of HS and intermittent reloading.** Rats of the three different ages were subjected to HS7 or HS14 to induce muscle atrophy. Rats subjected to HS lost body weight in the first few days, as reported previously (45), but this weight loss was similar for all age groups. Soleus and plantaris muscle weights at all three ages were decreased with HS7, and soleus muscle weight decreased further with HS14 (Fig. 1A and 2A). The mw/bw for soleus showed the same trend except that, in 32-mo-old rats, mw/bw did not significantly decrease with HS7 (Fig. 1B). Soleus MCSA followed the same pattern such that, at all three ages, MCSA was significantly decreased after HS14 (Table 1). Plantaris mw/bw did not decrease significantly with HS7 in 6- and 32-mo-old rats, but it was lower following HS14 at all ages (Fig. 2B). Therefore, HS is associated with a similar decrease in muscle mass in young as in old rats.

Intermittent reloading in young animals has been shown to decrease atrophy during HS (27), and, therefore, this intervention was utilized to investigate whether aging affects the ability of muscle to regulate its size. At 6 mo of age, intermittent reloading attenuated atrophy as measured by absolute muscle weight, mw/bw, and MCSA in soleus muscle, but, at 20 mo, only muscle weight and mw/bw showed significance (Fig. 1 and Table 1). In contrast, at 32 mo of age, intermittent reloading did not reduce atrophy in soleus muscle (Fig. 1 and Table 1). Therefore, intermittent reloading becomes ineffective in maintaining muscle mass as animals age.

Because the plantaris muscle did not show a significant attenuation of atrophy with intermittent reloading, even in young animals, we only used the soleus muscle for further experimentation.

**Changes in the Regulation of Myonuclear Number With Aging**

**Effect of age.** To explore mechanisms that may contribute to the age-dependent change in response to intermittent reloading, nuclear number in soleus muscle was investigated. Nuclear number per fiber was not different in control soleus muscles from rats at 6, 20, and 32 mo of age (Fig. 3A). This was surprising given that the muscles atrophy over this time period, suggesting that the myonuclear domain (i.e., amount of cytoplasm per nucleus) decreases with aging. Therefore, we determined the MCSA per nucleus as an estimate of myonuclear domain in the soleus muscle (Fig. 3B). Soleus muscles from old animals showed a lower MCSA per nucleus than those of young and middle-aged rats, indicating that the amount of cytoplasm per nucleus is lower at old age.

Although myonuclear number did not change with age, localization of nuclei within the fibers was different. In control soleus muscle from 32-mo-old rats, the number of centrally located nuclei was increased by fivefold compared with 6-mo-old soleus muscles from control rats (Fig. 4), suggesting ongoing regeneration that may include fusion of new nuclei into the muscle fibers of old animals. MyoD and myogenin, muscle-specific transcription factors known to be involved in satellite cell activation and differentiation, are elevated in muscles undergoing regeneration. In our study, the increase in centrally located nuclei at 32 mo of age was correlated with an increase in MyoD and myogenin mRNA abundance (Table 2), indicating that increased satellite cell activation is likely occurring at old age. To investigate this possibility, BrdU pellets
were implanted into the animals. Satellite cells are thought to proliferate before fusing into muscle fibers and will, therefore, be labeled with BrdU. The total number of BrdU-positive nuclei (both inside and outside the muscle membrane stained with dystrophin) was decreased at 20 and 32 mo of age in control muscles (Fig. 5A), indicating that proliferative capacity of cells present in the muscle decreases with aging. However, the number of BrdU-positive nuclei inside the muscle membrane is increased at 20 and 32 mo of age (Fig. 5B), suggesting that there is increased fusion of satellite cells into muscle fibers. Interestingly, BrdU-positive nuclei inside the muscle membrane were never centrally located in a muscle cell, but were always located peripherally (Fig. 6, arrowhead), suggesting that the centrally located nuclei in old muscle fibers were not derived from satellite cells that had undergone DNA replication in the time period during which the BrdU pellet was present (2 wk).

**Effect of HS.** As expected, myonuclear number decreased progressively with HS in adult (6-mo-old) soleus muscles, such that, after 14 days, myonuclear number was significantly decreased (Fig. 3A). However, in soleus muscle of 20- and 32-mo-old rats, no decrease in myofiber nuclei was found with HS (Fig. 3A), despite a similar decrease in muscle mass in 6-, 20-, and 32-mo-old rats in response to HS (Fig. 1A). MCSA per nucleus was decreased after HS14 in all of the age groups (Fig. 3B), but it was lower in old animals in every experimental condition. This suggests that the regulation of myonuclear domain size in both normal and unloaded animals is impaired with age. Similarly, the increase in MyoD and myogenin mRNA with HS14, as observed at 6 mo, was not seen at 20 and 32 mo of age (Table 2), suggesting that gene expression related to inactivity is altered with aging. The frequency of central nuclei did not change with the different experimental manipulations at any age (Fig. 4), indicating that centronucleation is a phenomenon likely not involved in the regulation of muscle size.

In 6-mo-old soleus muscle, there is a decline in total BrdU-labeled nuclei with HS, but, in muscles from the older two age groups, no significant decline in proliferative cells present in muscle was observed (Fig. 5A). In soleus muscle from 6-mo-old rats, HS did not change the number of BrdU-positive nuclei inside the muscle membrane (Fig. 5B), and, in soleus muscles from 20-mo-old rats, HS was actually associated with a decrease in BrdU-positive nuclei inside the muscle membrane, indicating that the control of satellite cell activation and fusion is dysregulated at an older age.

**Effect of intermittent reloading.** With intermittent reloading, there was no significant change in myonuclear number or MCSA per nucleus compared with HS only at any of the ages.
investigated (Fig. 3). In addition, centronucleation was not affected by intermittent reloading at any age (Fig. 4), indicating that the centrally located nuclei do not have a regulatory role in muscle size control. Interestingly, the number of BrdU-positive nuclei inside the muscle membrane was increased with intermittent reloading at 6 mo of age, suggesting that the fusion of satellite cells plays a role in the maintenance of myonuclear domain and likely in the attenuation of loss of muscle mass at this age. However, at 20 and 32 mo of age, the number of BrdU-positive nuclei inside the muscle membrane does not increase with intermittent reloading. This indicates that regulation of satellite cell activation and fusion with changes in muscle activity are altered with advancing age.

In addition, activity-dependent gene expression is also changed at old age, because gene expression of MyoD, which is attenuated by intermittent reloading at 6 mo of age, does not change at 20 and 32 mo of age (Table 2). These data indicate that, at older ages, the function of satellite cells is impaired, and this could contribute to the lack of response to intermittent reloading with aging.

**DISCUSSION**

It has been hypothesized that age influences the response of muscles to altered activity patterns (reviewed in Ref. 59). Indeed, muscles from old animals do not recover from atrophy as well as muscles from young animals (47, 68, 69) and do not respond to hypertrophic stimuli as well (9, 66). In this study, we showed that muscles from old animals were impaired in their response to an atrophy-reducing intervention, such as intermittent reloading. Atrophy (as measured by muscle mass) and myogenin mRNA abundance from soleus muscles after hindlimb suspension with and without intermittent reloading.

### Table 2. MyoD and myogenin mRNA abundance from soleus muscles after hindlimb suspension with and without intermittent reloading

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<tr>
<th></th>
<th>MyoD</th>
<th>Myogenin</th>
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<tr>
<td></td>
<td>6 mo</td>
<td>20 mo</td>
</tr>
<tr>
<td>Control</td>
<td>0.048±0.005</td>
<td>0.036±0.006</td>
</tr>
<tr>
<td>HS7</td>
<td>0.145±0.010</td>
<td>0.085±0.017</td>
</tr>
<tr>
<td>HS14</td>
<td>0.277±0.049†‡</td>
<td>0.070±0.021</td>
</tr>
<tr>
<td>HSIR14</td>
<td>0.154±0.013‡‡</td>
<td>0.093±0.034</td>
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Values are means ± SE in arbitrary density units. Significantly different from *6 mo and 20 mo, †control, ‡HS7, and ‡HS14: P < 0.05.
was reduced with intermittent reloading during HS in soleus muscle from young and middle-aged rats, but soleus muscle from old animals did not exhibit attenuation of muscle loss. In a previous study, Alley and Thompson (5) saw an attenuation of atrophy in soleus muscles from 30-mo-old rats. However, the rats in their study were hindlimb suspended for 7 days only, and the difference in age between 30 and 32 mo may be significant. The soleus muscle from 20-mo-old rats responded to intermittent reloading to the same extent as that from 6-mo-old rats with respect to muscle mass maintenance, and, therefore, the ability to respond to intermittent reloading is lost between 20 and 32 mo of age. However, the ability to maintain MCSA with intermittent reloading is also lost at 20 mo of age, and, therefore, it is possible that the amount of activity necessary to decrease atrophy is increased with advancing age, and a longer time period of intermittent reloading is warranted. Because the animals were observed continuously and exhibited the same amount of activity during reloading, the difference in response cannot be attributed to a difference in activity during the reloading period.

The regulation of myonuclear number in soleus muscle has been studied in adult animals. Hypertrophy is accompanied by an increase in myonuclear number, whereas skeletal muscle atrophy is associated with a decrease in myonuclei, such that the size of the myonuclear domain is relatively well maintained (reviewed in Ref. 4). Our laboratory has previously shown that changes in muscle size and myonuclear number after spinal cord transection and subsequent cycling exercise are very well correlated in young animals and that fusion of satellite cells is involved in this process (29). Only a few studies have investigated myonuclear number with aging. These studies suggested that myonuclear number does not decrease with advancing age (38, 63) and that there is no significant correlation between myonuclear number and fiber cross-sectional area in aged muscle, in contrast to young animals (64). Our data support these conclusions, as we observed no decrease in myonuclear number with aging and, consequently, a decrease in MCSA area per nucleus. The lack of a decrease in myonuclear number and the decrease in myonuclear domain could be explained if apoptotic nuclear loss was also decreased with aging. To the contrast, it has been shown that apoptosis actually increased with advancing age (23). The regulation of nuclear number with change in activity levels of muscles has not been investigated previously with respect to aging. In this study, we found that, at 6 mo of age, myonuclear number is decreased with unloading-induced atrophy, but, at older ages, this regulation of myonuclear number with atrophy is absent. Also, at old age, there is a dramatic increase in centrally located nuclei, regardless of activity level of the muscle, but these nuclei were not labeled with BrdU, indicating that they were not derived from previously replicated cells or that they had fused into the muscle fiber before the BrdU pellet was implanted.

Because satellite cells are required for full recovery from atrophy after HS (43) and also for hypertrophy (49, 53), we investigated the extent of satellite cell fusion with the lack of response to intermittent reloading with advancing age. In young animals (6 mo of age), the number of BrdU-labeled nuclei inside the muscle membrane was increased with intermittent reloading, indicating that proliferative cells fused with the muscle fiber. With advancing age, there was evidence of fusion of cells in control animals, but the increased fusion with intermittent reloading did not occur, indicating that the regulation of satellite cell function with changes in muscle size is impaired with aging. It has been shown that satellite cells isolated from old animals have a higher incidence of adipogenic differentiation (57), and, therefore, satellite cells of old animals may be less likely to contribute to new muscle nuclei, but instead contribute to an increase in fat. Moreover, it was shown recently that satellite cells from old animals failed to upregulate delta [notch ligand, involved in satellite cells activation (19)] in response to injury (18), indicating that there is a decrease in the activation of satellite cells at old age. Also, the total number of BrdU-positive nuclei, both inside and outside the muscle membrane, was decreased with advancing age to a level similar to that of young muscles after HS14, indicating that cellular proliferation in skeletal muscle decreased with aging. The identity of the BrdU-labeled cells in the interstitial space and the functional consequences of the decrease in proliferation with aging remain to be determined. Interestingly, BrdU-positive nuclei inside the muscle membrane were always located at the periphery of the fibers and not in a central position, even though there is an increase in centronucleation with aging. This suggests that there may be different subpopulations of cells that fuse into muscle fibers, either with or without dividing first to different locations within the muscle, or that the nuclei remain in the central position longer after fusing with the fiber at old age. Another possibility is that the centrally located nuclei do not arise from fused cells, but are nuclei resident within the muscle fiber that have become centrally located.

Previous studies have shown that gene expression of MyoD and myogenin is altered with advancing age. MyoD remained elevated after regeneration in muscles from old animals, in contrast to the restoration of normal expression in young and adult animals, suggesting that there was a decreased ability to regulate this myogenic factor (39). Also, it was shown that aging reduced the ability of muscles to increase MyoD protein levels during muscle unloading induced by HS (6). Our data confirm that, with advancing age, the regulation of gene expression of these transcription factors is altered. The increase in MyoD and myogenin mRNA with HS at 6 mo of age was not observed at older ages, and, more importantly, the attenuation of MyoD gene expression with intermittent reloading at young age was not seen at older ages. Therefore, it is likely that genes controlled by these factors are also dysregulated. Indeed, Patison et al. (47) recently showed that gene regulation during recovery from atrophy is altered in aged muscles, such that a growth factor (amphiregulin) was not upregulated, and a cell death-associated protein (clusterin) remained elevated in the old compared with the young. It remains to be determined whether these genes are regulated by MyoD or myogenin. The increase in MyoD and myogenin at old age could be indicative of an increase in satellite activation, although MyoD and myogenin have been shown to be expressed in both satellite cell as well as myofiber nuclei in old muscle (22).

Because we studied animals at three different ages, we were able to distinguish the progression of different processes with advancing age. It is of interest that the ability to maintain muscle mass is lost only at old age (32 mo), even though maintenance of MCSA, cell proliferative capacity, gene expression of MyoD and myogenin, and the regulation of myo-
nuclear domain are impaired at an earlier age (20 mo). Because muscle mass is determined by both the mass of the muscle fibers and the supporting structures, these findings imply that the regulation of the mass of supporting structures is changing with advancing age. Changes in cell proliferative capacity, gene expression, and regulation of myonuclear number are likely contributing to the altered regulation of muscle fiber size with advancing age, whereas mechanisms underlying the altered regulation of mass of supporting structures remain to be elucidated.

In summary, attenuation of the loss of muscle mass during an atrophy-inducing event is impaired with aging, and we suggest that the decrease in proliferation and the changed function of satellite cells with advancing age contributes to the impaired response of muscles to changes in activity level.

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