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Effects of aging on basement membrane of the soleus muscle during recovery following disuse atrophy in rats

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ABSTRACT

Aging is known to lead to the impaired recovery of muscle after disuse as well as the increased susceptibility of the muscle to damage. Here, we show that, in the older rats, reloading after disuse atrophy, causes the damage of the muscle fibers and the basement membrane (BM) that structurally support the muscle fibers. Male Wistar rats of 3-(young) and 20-(older) months of age were subjected to hindlimb-unloading for 2 weeks followed by reloading for a week. In the older rats, the soleus muscles showed necrosis and central nuclei fiber indicating the regeneration of muscle fibers. Furthermore, ectopic immunoreactivity of collagen IV, a major component of the BM, remained mostly associated with the necrotic appearance, suggesting that the older rats were impaired with the ability of repairing the damaged BM. Further, after unloading and reloading, the older rats did not show a significant alteration, although the young rats showed clear response of *Col4a1* and *Col4a2* genes, both coding for collagen IV. In addition, during the recovery phase, the young rats showed increase in the amount of *Hsp47* and *Sparc* mRNA, which are protein folding-related factor genes, while the older rats did not show any significant variation. Taken together, our findings suggest that the atrophic muscle fibers of the older rats induced by unloading were vulnerable to the weight loading, and that attenuated reactivity of the BM-synthesizing fibroblast to gravity contributes to the fragility of muscle fibers in the older animals.

1. Introduction

Skeletal muscle mass and functionality can be changed in response to physical activity. Mechanical overload like resistance exercise improves the muscle mass and the muscular strength (Schiaffino et al., 2013) and, on the other hand, disuse causes muscle atrophy (Thomason and Booth, 1990). After reloading, compared with the young animals, the older animals show delayed recovery from disuse muscle atrophy (Hwee and Bodine, 2009; White et al., 2015). The reason for the sluggish recovery in the older animals, however, is poorly understood. Previous studies have revealed age-related dysfunction in the recovery process from disuse atrophy. Decreased function of satellite cells with aging was suggested to contribute to the impaired response of muscle to reloading (Gallegly et al., 2004). During the muscle recovery, some molecules necessary for protein synthesis shows attenuated responses such as delay in the activation of Akt (Hwee and Bodine, 2009), or a decreased phosphorylation of $p70^{S6K}$ in the muscle fibers of the aged animals (Morris et al., 2004).

Basement membrane (BM) is a layer of extracellular matrix material, which coats muscle fiber as a static structure that provides mechanical support (Sanes, 2003). Previous studies have shown that BM plays a critical role in maintaining muscle structure and function. Deficiency of the molecular components of BM results in muscular dystrophy associated with fragility of muscle fibers (Girgenrath et al., 2009; Labelle-Dumais et al., 2011). Therefore, the slow recovery of muscles in the older animals can possibly be attributed to the malfunction of BM.

The soleus muscle is an anti-gravity muscle, which has similar characteristics of slow-twitch postural muscle and is used for the study of disuse atrophy induced by hindlimb unloading. The soleus muscles of rats showed atrophy after unloading of hindlimb and recovery of mass after reloading such as weight bearing (Oishi et al., 2008). Flück et al. (2003) have shown that reloading of atrophied rat soleus muscle induces tenascin-C, a marker of damaged muscle fibers, suggesting that

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reloading of body weight would be a mechanical stress that damages atrophied muscle fibers. Here, we hypothesized that, after unloading, muscle fibers of the older animals are at high risk of damage by reloading. In the present study, we have investigated the effects of aging on the recovery process after the periods of hindlimb unloading followed by reloading. We have compared the soleus muscles of the young rats with the older rats to observe the morphological and molecular changes of muscle fiber and BM in the recovery process from disuse atrophy.

2. Materials and methods

2.1. Animals

Male Wistar rats ($n = 32$) were obtained from Japan SLC (Shizuoka, Japan). Three-month-old rats were used as young rats and 20-month-old rats were used as older rats. All animals were housed in individual cages and allowed free access to food and water constantly. The environmental conditions were maintained at 22 ± 2 °C and a 12:12 h light: dark cycle.

This study was approved by the Committee of Animal Care and Use of the Kindai University Faculty of Medicine (KAME-26-049). All experimental procedures were conducted in accordance with institutional guidelines for the use of experimental animals.

2.2. Experimental protocol

The young and older rat groups ($n = 16$ each) were divided into 4 groups each ($n = 4$ per group): non-unloaded ambulatory controls (Cont), hindlimb unloaded for 14 days after unloading (HU14d). After the unloading, the rats were allowed to move freely in the cage for one (RE1d) or seven (RE7d) days.

2.3. Hindlimb unloading and reloading

Hindlimb unloading was performed as previously described with minor modifications (Morey-Holton and Globus, 2002). Briefly, hindlimbs of rats in the HU14d, RE1d, and RE7d groups were prevented from bearing weight by tail suspension and the rats were allowed free access to food and water by their forelimbs during the hindlimb unloading. The unloading continued for 14 days, after which the rats in the RE1d and RE7d groups were released from the tail suspension. Hindlimbs of the rats were reloaded in order to recover their muscle atrophy for both RE1d and RE7d groups.

2.4. Sampling

At the end of the experimental period, rats were anesthetized using sodium pentobarbital, after which the soleus muscles were removed and weighed. A part of the soleus muscle belly of each rat was excised and preserved in RNAlater (Thermo Fisher Scientific, Hanover Park, IL) and other parts of the soleus muscles were frozen immediately in isopentane, cooled in dry ice, and stored at − 80 °C for further analyses.

2.5. Histochemical analyses

Summation of extracellular matrix, muscle fibers, and tissue fluid is evaluated by the muscle wet weight. Therefore, in order to evaluate the muscle fibers alone, fiber cross sectional area (FCSA) that measures the cross-sectional area of the muscle fiber was adopted. Transverse tissue sections (10-μm thickness) were cut from the middle part of the soleus muscle belly using a cryostat (CM1950; Leica, Wetzlar, Germany) at − 25 °C and were mounted onto amino silane-coated glass slides. Then, to know the ratio of type I and type II fibers and fiber type specific FCSA, an ATPase staining was carried out using a calcium method (Brooke and Kaiser, 1970). In short, the sections were treated with myofibrillar adenosine triphosphate (ATPase) staining after alkaline pre-incubation (pH 10.7). The sections were then washed in 1% $CaCl_2$, reacted with 2% $CoCl_2$ and finally washed in 0.005 M sodium barbital. After washing with distilled water, the sections were visualized by staining with 2% ammonium sulfide. After staining, the sections were dehydrated with a graded series of ethanol rinsing, immersed in xylene and embedded in Entellan (Merck, Darmstadt, Germany). FCSA, the number of muscle fibers, and histopathologic findings were analyzed to investigate the effect of hindlimb unloading and reloading in the young rats and older rats. Sections were stained using Hematoxylin and Eosin (HE) and observed under microscope. The FCSA was measured using Image J software (NIH, Bethesda, Maryland).

2.6. Electron microscopy

Longitudinal tissue sections (1-mm thickness) were cut from the middle part of the soleus muscle belly at − 25 °C. The sections were fixed with 4% paraformaldehyde/2% glutaraldehyde and then treated using osmium tetraoxide, after which they were dehydrated using a series of ethanol gradients. The sections were finally embedded in Epon. Ultrathin sections (90-nm thickness) were cut with an ultra-microtome and stained with 4% uranyl acetate and 1% lead citrate. The sections were analyzed by transmission electron microscopy (HT7700; Hitachi, Tokyo, Japan).

2.7. Immunohistochemical analysis

Transverse sections were fixed in 4% paraformaldehyde and were rinsed with PBS (pH 7.4). The sections were then bleached with 3% H_2O_2, rinsed with PBS, and incubated for 1 h at 4 °C in PBS containing 1% normal goat serum and 0.3% Triton X-100. The sections were then incubated for 24 h at 4 °C in rabbit polyclonal anti-collagen IV antibody (ab6586; Abcam, Cambridge, MA) diluted 1:500 in PBS containing 0.3% Triton X-100. The sections were subsequently incubated for 1 h at room temperature in biotinylated anti-rabbit immunoglobulin G (IgG) (Vectastain ABC kit; Vector Laboratories, Berlingame, CA) diluted 1:1000 in PBS, after which they were incubated in avidin-biotin complex (Vectastain ABC kit) for 1 h at 4 °C. After rinsing with PBS, the sections were washed with Tris-HCl buffer (pH 7.4) and incubated with diaminobenzidine (0.035% DAB) in Tris-HCl buffer (0.003% H_2O_2) for 15 min at room temperature. After the DAB reaction, the sections were also stained with Hematoxylin and dehydrated with a graded series of ethanol rinses, immersed in xylene, and embedded in Entellan (Merck, Darmstadt, Germany).

2.8. Quantitative PCR

Total RNA extracts of the soleus muscle belly saved in RNAlater were prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan) with random primers. Quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan). The quantity of each target gene PCR product was normalized to *Gapdh* levels and up- or down-regulation was calculated as fold change relative to the Cont group in the young rats. The oligonucleotide primer sets used in these PCR analyses were as follows:

Col4a1, 5′-ATGCCAGGAAGAGCAGGAAC-3′ (Forward) and 5′-CGACTACCAGGAAAGCCAACTC-3′ (Reverse);

Col4a2, 5′-ACAGGGACCCTCTGGAACTG-3′ (Forward) and 5′-CGATATTTGTCACGGTCCTCTTT-3′ (Reverse);

Hsp47, 5′-CGCAGCAGTAAGCAACACTACA-3′ (Forward) and 5′-TCCACATCCTTGGTGACCTCT-3′ (Reverse);

Sparc, 5′-GACTACATCGGACCATGCAAATAC-3′ (Forward) and 5′-GGTTGTTGCCCTCATCTCTCT-3′ (Reverse);

Mmp14, 5′-GGATACCCACTTTGATTCTGCTG-3′ (Forward) and 5′-