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Changes in cross sectional area per myonucleus on mice *soleus* muscle during one week of hindlimb suspension reinforce the concept of myonuclear domain

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Objective: The effects of hindlimb unweighting by tail suspension on mice's soleus muscle cross-sectional area (CSA) and myonuclei content were studied. ***Design:*** Five groups of male Charles River CD 1 mice (n=10 per group) were assigned to varying hindlimb suspension (HS) periods: control (cont), 1 (1HS), 2 (2HS), 3 (3HS) and 8 (8HS) days. Immediately after the suspension period, the soleus muscles were removed in order to assess morphometry data. The CSA and number of myonuclei were determined in a total of 5011 fibers (1098, 793, 1363, 1281, 1276 fibers for the cont, 1HS, 2HS, 3HS, 8HS groups, respectively) from different areas in the mid-portion region of the soleus muscle, and the CSA per myonucleus ratio was calculated. ***Results:*** The results showed that the removing of weight bearing had a negative impact on muscle fiber CSA (1686 ± 516 μm^2, 1506 ± 470 μm^2, 1235 ± 340 μm^2, 1277 ± 412 μm^2 and 1075 ± 356 μm^2 in the cont, 1HS, 2HS, 3HS and 8HS groups, respectively) and myonuclei number per fiber (1.91 ± 1.17, 1.94 ± 1.30, 1.55 ± 1.01, 1.48 ± 1.00 and 0.83 ± 0.76 in the cont, 1HS, 2HS, 3HS and 8HS groups, respectively). The CSA per myonucleus ratio was 993 ± 522 μm^2, 854 ± 443 μm^2, 815 ± 374 μm^2, 881 ± 442 μm^2 and 957 ± 367 μm^2 in the cont, 1HS, 2HS, 3HS and 8HS groups, respectively. Thus, there were no significant differences between the control and 8HS groups. ***Conclusion:*** The morphological data is consistent with the hypothesis that, despite the muscle fiber atrophy induced by HS, the CSA per myonucleus ratio is apparently maintained, reinforcing the concept of myonuclear domain.

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Key Words: skeletal muscle atrophy; simulated weightlessness; myonuclear domain; myonuclear number; skeletal muscle plasticity

INTRODUCTION

The skeletal muscle fiber is a syncytium where various myonucleus share their products. This particularity, associated with the fact that in single nuclear cells the nucleus controls the surrounding cytoplasm, has led to the concept of myonuclear domain (MND), which is the theoretical volume of cytoplasm within the myofiber regulated by the gene products of a single myonucleus (1, 2). This concept is reinforced by several studies reporting that the myonuclei number changes with myofiber volume modifications, as an attempt to maintain a constant relationship between the area of cytoplasm per myonucleus (3). Indeed, several studies analysing the muscle fibers growth and hypertrophy conclude that the myonuclei number increases during the enlargement of the muscle fiber (4-7). Conversely, studies evaluating the skeletal muscle atrophy process reported a proportional reduction between the myonuclei number and the muscle fiber size (8-10). Nevertheless, different results have been also reported. Regarding the skeletal muscle hypertrophy process, one study using isolated fibers from mice has reported that the cytoplasmic enlargement during muscle hypertrophy is followed by a decrease in the myonuclear number (11). Throughout the skeletal muscle atrophy process, various studies indicate that the myonuclear number is not synchronized with the myofiber size (12-17). In a study where atrophy was induced by 28 days of HS, a significant decrease in CSA and a significant increase in myonuclei density (myonuclei per mm of fiber)

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were found (16). In a recent in vivo study, the results of different atrophy methods (denervation, nerve impulse block and tenotomy for mechanical unloading) essentially led to the same conclusion: atrophy was not accompanied by the proportional loss of myonuclei (17). Methodological flaws, however, may be the main reason for the occurrence of these disparate results, and therefore for the controversy generated regarding the relationship between skeletal muscle fiber size and myonuclei number.

During the skeletal muscle atrophy process there is an increased wasting or loss of muscle mass and usually a decrease in the myofibers cross-sectional area (CSA) along with the disappearance of myonuclei, since the transcriptional and translational demands placed on myonuclei are attenuated (reviewed in 18). The slow-twitch soleus muscle is preferentially affected in the hindlimb in most atrophy-inducing mechanisms: hindlimb suspension (HS) (19, 20), spaceflight (8, 21), bed rest (22) and denervation (14, 23). Atrophy in this muscle, responsible for maintaining posture and counteracting the effects of gravity, is emphasized because unweighting preferentially affects it compared with other calf muscles – which are mainly fast-twitch muscles (reviewed in 24).

In the present study, we have investigated the effects of HS on mice soleus muscle CSA and myonuclei number. Changes in myofiber morphology and number of myonuclei were monitored within groups of mice with different periods of HS. We hypothesized that HS-induced changes in fiber CSA are associated with decreased number of myonuclei so that the MND size is maintained. The results of this study may contribute to clarifying whether MND size is maintained during muscle fiber atrophy (induced by HS) and thus whether the reduction of myonucleus number is proportional to the reduction in fiber CSA, which is the underlying key question.

MATERIAL AND METHODS

Animal model and experimental design

A total of 50 male Charles River CD 1 mice (6-8 weeks old, 30-35 g body weight) were used for this study. All animals were housed in collective cages (two mice per cage) at normal atmospheric conditions (21-22°C; 50-60% humidity) in an inverted 12-h light/dark cycle and received commercial food for rodents and water ad libitum. The animals were randomly assigned in equal numbers (n=10 for each group) to one of five groups. In four groups, the animal were hindlimb-suspended to prevent ground support and loading for 1 (1HS), 2 (2HS), 3 (3HS) and 8 (8HS) days, by use of a tail harness [25]. The forelimbs maintained contact with the cage floor, which permitted a full range of motion for the mouse. The remaining ten animals comprised the control group (cont). After the experimental period, the animals of each group were killed by a cervical dislocation and the soleus muscles of both hindlimbs were completely excised and processed for light microscopic (LM) evaluation. All the experiments were performed after approval of the local ethics committee and in accordance with the Guide for the Care and Use of Laboratory Animals.

Tissue preparation for LM evaluation

Small sections of tissue (≈ 1 mm^3) from the mid-portion of the soleus were immersed in a solution containing 4% paraformaldehyde and 2.5% sucrose diluted in a phosphate buffer at pH 7.2 for 2 hours. Rinsing was performed with 0.1 and 0.2 M cacodylate buffer after which the tissue sections were dehydrated with graded ethanol. The pieces were then fixed [4% (v/v) buffered paraformaldehyde] by diffusion during 24 hours and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xilene was used in the transition between dehydration and impregnation. Serial sections (5µm of thickness) of the paraffin blocks were cut by a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohol concentrations, finishing in phosphate buffered saline solution prepared by dissolving Na2HPO4 (1.44 g), KH2PO4 (0.24 g), NaCl (8 g), KCl (0.2 g) in 1 litre of deionised water and adjusting pH to 7.2. Deparaffinised sections were stained with haematoxylin-eosin and analysed in the photomicroscope (Zeiss Axioplan 2, Carl Zeiss Germany). Photographs of soleus cross-sections of the control and groups 1HS to 8HS were digitalized and analysed with the NIH ImageJ (Image Processing and Analysis in Java, USA) software. The number of fibers analysed, for CSA and myonuclei number per fiber quantification, was 1098, 793, 1363, 1281, 1276 in the control, 1HS, 2HS, 3HS and 8HS groups, respectively.

Statistical analysis

Normality analysis was performed with the Kolmogorov-Smirnov test. Mean and standard deviations were calculated for all variables in each of the experimental groups. For the CSA per myonucleus ratio a percentile distribution (presented in boxplot graphics) was calculated for all groups. The One-Way ANOVA was used to test differences between different moments of the experimental protocol. The Statistical Package for the Social Sciences (SPSS Inc. Version 17.0) was used for all analyses. The significant level was set at $\alpha = 0.05$.

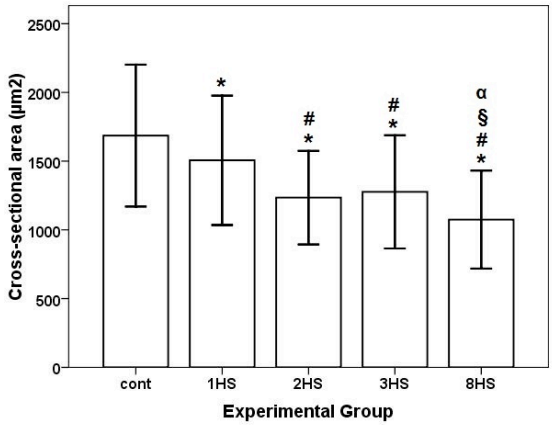

Figure 1. Cross-sectional area in soleus muscle during 8 days of hindlimb suspension. Values are mean ± standard deviation (*, #, §, α, P < 0.05 versus control, 1HS, 2HS, and 3HS group, respectively).

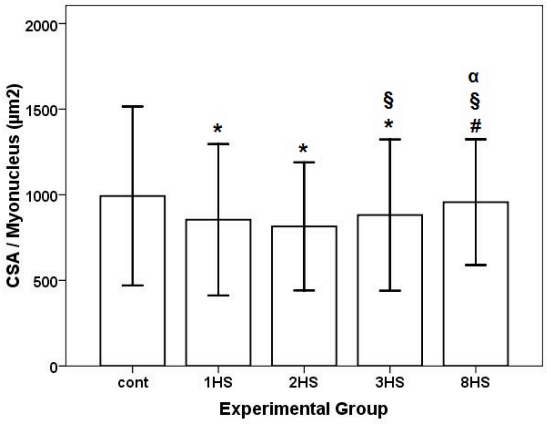

Figure 2. Cross-sectional area per myonuclei in soleus muscle during 8 days of hindlimb suspension. Values (mean ± standard deviation) are presented in µm2 (*, #, §, α, P < 0.05 versus control, 1HS, 2HS, and 3HS group, respectively).

RESULTS

Effects on muscle CSA

The application of HS resulted in a significant reduction in the fiber size of soleus (figure 1). Compared to control values (1686 ± 516 µm2), the CSA decreased 10.7% (1506 ± 470 μm^2), 14.3% (1235 ± 340 μm^2), 16.7% (1277 ± 412 μm^2) and 36.3% (1075 ± 356 μm^2) in the 1HS, 2HS, 3HS and 8HS groups, respectively.

Effects on myonuclei number per fiber

During the experimental period, a significant reduction in the myonuclei number per fiber of the soleus muscle was noticed (Table 1). Beside fewer myonuclei per fiber, we also found an increased number of fibers without myonuclei in the cross section. Compared to the control values, the percentage of fibers without myonuclei increased 2.7%, 5.9%, 6.3% and 26.6% in the 1HS, 2HS, 3HS and 8HS groups, respectively.

Changes in the CSA per myonucleus ratio

As can be seen in figure 2, compared to the control group (993 ± 522 μm^2), the experimental period resulted in a significant decrease on CSA per myonucleus in the soleus muscle in the 1HS (854 ± 443 μm^2), 2HS (815 ± 374 μm^2) and 3HS (881 ± 442 μm^2) groups. Nevertheless, there were no significant differences between the control and the 8HS (957 ± 367 μm^2) group. Compared to the control values, the CSA per myonucleus was in 14%, 17.9%, 11.2% and only 3.6% in the 1HS, 2HS, 3HS and 8HS groups, respectively. Figure 3 shows the distribution of the CSA per myonucleus ratio for all the experimental groups. The median values are 838 μm^2, 716 μm^2, 726 μm^2, 775 μm^2, and 928 μm^2 for the cont, 1HS, 2HS, 3HS and 8 HS groups, respectively. 50% of the CSA per myonucleus ratio values are between 602 μm^2 and 1287 μm^2 in the control group, 523 μm^2 and 1093 μm^2 in the 1HS group, 518 μm^2 and 1053 μm^2 in the 2HS group, 536 μm^2 and 1146 μm^2 in the 3HS group and 667 μm^2 and 1210 μm^2 in the 8HS group.

DISCUSSION

The methodology used in the present study focuses on the quantification of CSA and myonuclei numbers in the soleus muscle fibers, which could give some insight regarding the maintenance, removal or increase of myonuclei per fiber and therefore about the MND during skeletal muscle atrophy induced by 8 days of HS.

Supporting the process of muscle atrophy, our morphometric analysis showed that mice HS induced a significant decrease in the mean fiber CSA of soleus muscle (fig. 1), which is in agreement with prior studies (16, 19, 20, 26, 27). This decline in CSA was more pronounced after the first day and then again between the third and the eighth days of suspension. Between the 2HS and 3HS groups there were no significant differences in the mean CSA (1235 μm^2 versus 1277 μm^2, respectively). These results suggest that the earlier atrophy-induced alterations are mainly derived from biochemical/molecular events, which does not seem to be paralleled by the cellular rate of myonuclei loss, despite the relationship between the two events.

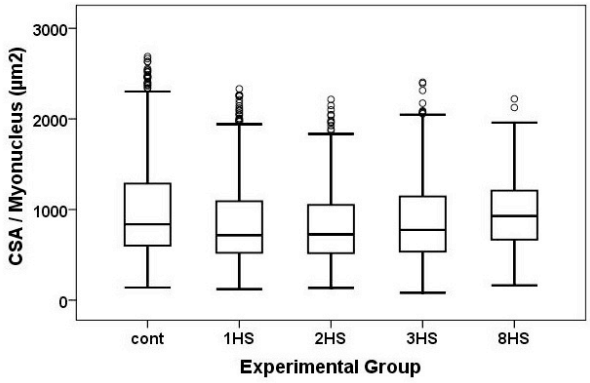

Figure 3. Cross-sectional area per myonucleus ratio distributions during the experimental protocol; O, outliers; upper limit of the box, 75th percentile value; lower limit of the box, 25th percentile value; horizontal line through box, median value.

However, this initial increased rate of proteolysis and/or decreased protein synthesis (reviewed in 28, 29) seems to be balanced by the rate of nuclei loss by apoptosis or necrosis after approximately 48 hours of inactivity (as we can see comparing the CSA of the 2HS and 3HS groups).

As shown in table 1, induced inactivity with HS has resulted in a significant decrease in the myonuclei number per fiber. These results are consistent with previous studies (19, 30) but differ from others reporting a myonuclei number increase (16, 31). Our results show that this significant decrease in the myonuclei number per fiber was only verified after 2 days of induced-atrophy. This alteration may be explained if we consider that various studies described that the number of myonuclei undergoing apoptosis significantly increases in the early stages of HS (20, 32) and hindlimb immobilization (10) and that these events were more prominent between the second and the third days of muscle inactivity.

Table 1. Myonuclei number per fiber and percentage of fibers without myonuclei in soleus muscle during 8 days of hindlimb suspension.

Groups	Myonuclei per fibre	Percentage of fibres without myonuclei
cont	1.91 ± 1.17	9.7%
1HS	1.94 ± 1.30	12.4%
2HS	1.55 ± 1.01* #	15.6%
3HS	1.48 ± 1.00* #	16%
8HS	0.83 ± 0.76* # § α	36.3%

Myonuclei number express the ratio between the total of myonuclei nuclei present in each group and the total of fibers analysed (*, #, §, α, $P < 0.05$ versus control, 1HS, 2HS, and 3HS group, respectively).

The present study demonstrates that this experimental protocol induces fibers alterations in muscle CSA that seem to be proportional to the changes in the myonuclei number per fiber. In fact, as we can see in figure 2, there are no significant differences in the fiber CSA per myonucleus ratio between the control and the 8HS groups, supporting the concept of the MND, and the idea that its maintenance is verified under conditions of muscle atrophy, as reported in previous studies (8, 22, 23). However, our results showed (figure 2) that this ratio is not always preserved. This outcome may be due to the skeletal muscle adaptation to the atrophy process. As we previously mentioned, there is an increased proteolysis and a decreased protein synthesis in the skeletal muscle induced by its inactivity, and both mechanisms seems to start immediately after the onset of muscle unloading (33). This catabolic system seems to be faster than that affecting the myonuclei number decrease by apoptosis. As can be seen in figure 3, the distribution of the fiber CSA per myonucleus ratio shows that the 1HS, 2HS and 3HS groups are positive-skewed; i.e., the majority of the observations are concentrated in the bottom of the graphic but there are some observations on the top of it, indicating that there are some fibers with a large CSA and few myonuclei. However, in the 8HS group this ratio imbalance is attenuated and therefore, no significant differences were found in the CSA per myonucleus ratio between the control and the 8HS group (as shown in figure 2). We choose to include figure 3 because displaying data in boxplot form allows for conveying at least four parameters of a distribution in a single, visually enjoyable and informative display. In addition, reporting only single values, such as means and standard deviations, can be very uninformative or even misleading.

Nevertheless, there are numerous studies demonstrating that this CSA per myonucleus ratio is not preserved in many atrophy-inducing mechanisms (14-17, 31). As referred to in the introduction, this discrepancy in the results may be a consequence of some methodological inaccuracies, but it can be also related to the fact that most of the studies emphasize that muscle undergoes changes, i.e., during atrophic or hypertrophic stimulus. Our results suggest that during the dynamic phase of muscle adaptation, the loss in muscle CSA is disproportionate to the rate of loss of myonuclei. Other reasons for the inconsistency of the results is the fact that most studies do not report the number of fibers analysed (15, 16, 31), and calculate the mean myonuclei number per fiber (analysing several points along the fiber segment), assuming that myonuclei density is uniform across the entire fiber and the number of analysed fibers may be insufficient (200 to 250 fibers) (8, 14).

Our results are based on the direct observation of

sections of more than 5000 fibers. Nevertheless, this study has some limitations that need to be acknowledged. In our methodology, we did not use any strategy to ensure that the satellite cells present in the soleus muscle were identified and were not erroneously counted as myonuclei, as in other studies in which the tissue sections are strained with antibodies against dystrophin (8, 17). Although the numbers of this type of cell are small, compared with those of true myonuclei – as shown in one study of soleus muscle fibers indicating that a maximum of 4-5% of the total muscle nuclei are satellite cells (34) – it is possible that minor errors in the myonuclei number count occurred.

In conclusion, the present study demonstrates that HS causes an imbalance between the soleus muscle fibers CSA and myonuclei number per fiber in the first days of suspension. Further, the results suggest that after 8 days of HS the muscle fibers CSA per myonucleus ratio are the same as those observed in the control group, emphasizing the theoretical concept of MND and its maintenance along changes in muscle size. To our knowledge, this is the first study reporting this amount (number of fibers analysed) of data concerning HS induced atrophy.

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