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No change in myonuclear number during muscle unloading and reloading

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Bruusgaard JC, Egner IM, Larsen TK, Dupre-Aucouturier S, Desplanches D, Gundersen K. No change in myonuclear number during muscle unloading and reloading. *J Appl Physiol* 113: 290–296, 2012. First published May 10, 2012; doi:10.1152/japplphysiol.00436.2012.—Muscle fibers are the cells in the body with the largest volume, and they have multiple nuclei serving different domains of cytoplasm. A large body of previous literature has suggested that atrophy induced by hindlimb suspension leads to a loss of "excessive" myonuclei by apoptosis. We demonstrate here that atrophy induced by hindlimb suspension does not lead to loss of myonuclei despite a strong increase in apoptotic activity of other types of nuclei within the muscle tissue. Thus hindlimb suspension turns out to be similar to other atrophy models such as denervation, nerve impulse block, and antagonist ablation. We discuss how the different outcome of various studies can be attributed to difficulties in separating myonuclei from other nuclei, and to systematic differences in passive properties between normal and unloaded muscles. During reload, after hindlimb suspension, a radial regrowth is observed, which has been believed to be accompanied by recruitment of new myonuclei from satellite cells. The lack of nuclear loss during unloading, however, puts these findings into question. We observed that reload led to an increase in cross sectional area of 59%, and fiber size was completely restored to the presuspension levels. Despite this notable growth there was no increase in the number of myonuclei. Thus radial regrowth seems to differ from de novo hypertrophy in that nuclei are only added during the latter. We speculate that the number of myonuclei might reflect the largest size the muscle fibers have had in its previous history.

apoptosis; atrophy; hypertrophy; myonuclei; skeletal muscle

FORCE IS LARGELY PROPORTIONAL to the number of contractile filaments over the cross section of muscle. Changes in a muscle's ability to generate force will largely occur by alterations in the cross-sectional area (CSA) of each fiber, although changes in the number of muscle fibers (10) or the density of contractile material (9, 13) may also contribute. Thus fiber hypertrophy and atrophy are the central mechanisms for alterations in muscle strength in health and disease. In particular, muscle atrophy is a major health problem related to inactivity, injury, disease, aging, and medication; and maintaining and regaining muscle strength is an important factor for life expectancy and quality (27). The most important factor in maintaining or regaining muscle force is nerve-evoked contractile activity (32, 33). There is so far no practical remedy for inactivity-related atrophy other than by restoring or increasing physical activity (31).

Changes in fiber size are achieved by regulating three major conditions: *1*) the number of cell nuclei within the muscle fibers (myonuclei); *2*) the rate of protein synthesis for each nucleus; and *3*) the rate of protein degradation (33). Historically, it has been believed that a nucleus can support a certain volume of cytoplasm and, consequently, that the so-called "karyoplasmatic" ratio is constant (62). Modern literature has extended this idea by demonstrating that in multinucleated muscle fibers each nucleus seems to synthesize protein for a local domain in the vicinity of that nucleus both in vitro (36, 52) and in vivo (35). On the other hand, there is not always a good correlation between CSA and the number of nuclei (16, 17, for review of older literature see 34, 71).

In agreement with the idea of constant myonuclear domains, hypertrophy is accompanied by recruitment of new nuclei by satellite cells fusing with the preexisting muscle fiber syncytia as shown ex vivo using histological methods (4, 5, 18–20, 26, 30, 41, 44, 45, 50, 51, 54, 55, 58, 73), and also more recently using in vivo imaging of myonuclei (15).

The opposing popular idea that during atrophy, nuclei are lost by apoptosis (1–3, 6–8, 12, 21, 23–25, 28, 38, 40, 43, 57, 59–61, 63, 66, 70, 74), was, however, recently refuted for atrophy caused by denervation, nerve impulse block, or antagonist ablation using direct observation by in vivo imaging of myonuclei (14, 15, 34).

The first goal of the present study was to reinvestigate the alleged loss of myonuclei during hindlimb suspension with improved methods, and we report that nuclei are not lost. Second, since this observation questioned the idea that nuclei are added during muscle growth we wanted to reinvestigate if nuclei are added during reload. If nuclei were recruited into myofibers under such conditions, this would imply that this process is related to fiber growth per se. If, on the other hand, nuclei are not recruited during regrowth to original size, it might imply that nuclear addition is a phenomenon related to muscle fibers exceeding their maximal previous size. The present data favor this theory.

MATERIALS AND METHODS

Animal experiments. Pathogen-free 12-wk-old female Wistar rats weighing ~200 g from Charles River were housed in a temperature-controlled room (24 ± 2°C) with a 12:12-h light/dark cycle. After 1 wk of acclimatization, rats were hindlimb unloaded for a period of 14 days in individual cages using Morey's tail-suspension model (49). Adhesive tape was wrapped around the tail and connected to a pulley by a plastic bar. Rats were able to move in a 360° arc with their forelimbs, thus allowing exercise and access to food and water, although the hindlimbs were not load bearing. The animals were suspended at ~20° head-down tilt angle to minimize lordosis. Animals caged normally during the experimental periods served as controls. After 2, 4, and 14 days of suspension, one group was euthanized while another was allowed to perform normal cage activity for a subsequent 14 days, permitting reloading of the hindlimb muscles. At the endpoints, rats were anesthetized with halothane. Soleus muscles were excised, weighed, and frozen in a slightly stretched state as to maintain a length near to resting length in melting isopentane and stored at −80°C until sectioned.

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The animal experiments were approved by the Institutional Animal Care and Use Committee from the University Lyon 1, following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe number 123, Strasbourg, 1985).

Histology. Soleus muscles were cryosectioned at 10 μm and blocked in 1% bovine serum albumin, then stained with an anti-dystrophin monoclonal antibody from Sigma at a 1:200 dilution. DNA fragmentation associated with apoptosis was detected subsequently by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL), using the TdT-FragEL detection kit (EMD Biosciences) according to the manufacturer's instructions. Nuclei were finally costained using Hoechst dye 33342 (Invitrogen) at a concentration of 5 μg/ml in PBS. Slides were observed in an Olympus fluorescence microscope with a 60× 0.9 NA water immersion objective. Nuclei were counted as TUNEL-positive only if colocalization of the Hoechst dye and TUNEL-staining was observed.

As discussed previously (14, 34), we interpreted nuclei with their geometric center outside the dystrophin "ring" to either belong to satellite or stromal cells. Conversely, we considered nuclei that had their geometric center inside the inner rim of the dystrophin "ring" to be myonuclei (Fig. 1).

Fiber CSA was measured by manually encircling each fiber using the lasso tool in Adobe Photoshop CS5.

Single fiber analysis. Myonuclei of single soleus fibers were investigated by in vivo imaging as described previously for mice (15), but using a different camera and image-acquisition system. Single soleus muscle fibers from male Wistar rats were injected in situ with a phosphorothioated backbone, random 17-mer, TRITC-labeled oligonucleotide. A random sequence was used, and a BLAST analysis confirmed that the sequence was not represented in the genome. The labeled DNA injections were performed essentially as described previously (69). Borosilicate 1.0/1.5-mm glass micropipettes with a filament (World Precision Instruments, Sarasota, FL) were pulled on a laser-heated puller (P2000, Sutter Instruments, Novato, CA) and beveled on an audiocassette tape moving at playing speed. The DNA was dissolved in a buffer containing 10 mM NaCl, 10 mM Tris (pH 7.5), 0.1 mM EDTA, and 100 mM potassium gluconate. The filled pipettes were placed in a micromanipulator (Sutter Instruments) and used to penetrate the cells. The solution was ejected by pulses of pressurized air (100–150 hPa) lasting 20 ms and delivered at 500-ms intervals by a PV820 pneumatic pico-pump (World Precision Instruments). A constant holding pressure of ~1 hPa was also applied to prevent the solution from moving back into the pipette between pulses. The injected oligonucleotides were rapidly taken up by the myonuclei, probably by active transport (37). Both ultrastructural (56) and electrophysiological evidence (11) indicates that there are no gap junctions between the satellite cells and their muscle fibers or between muscle fibers. The labeled DNA was never seen to leak out of the fibers or to label more than one fiber; thus only the nuclei confined within one plasma membrane were labeled.

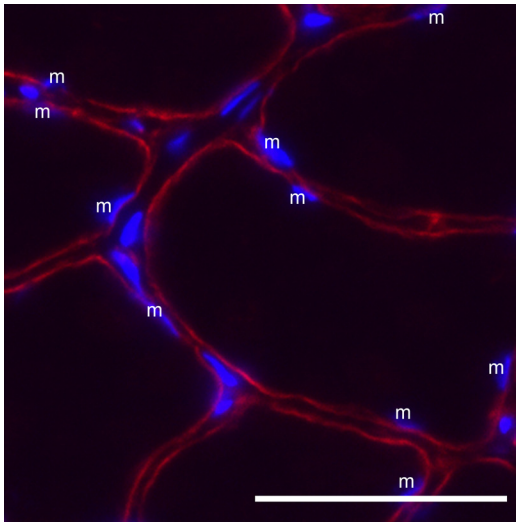

Fig. 1. The inclusion criteria for myonuclei seem to be critical. We defined nuclei that had their mass center inside the inner rim of the dystrophin "ring" to be myonuclei myonuclei (labeled m) while the rest were nonmyonuclei (unmarked). The sections are stained with antibodies against dystrophin (red) and Hoechst 33342 against DNA (blue). Scale bar is 50 μm.

Twenty minutes after the injection, fiber segments of 250–1,000 μm were analyzed by acquiring images in different focal planes, each 5 μm apart, on an Olympus BX-50WI compound microscope with a 20× 0.3 NA long working distance water immersion objective. All images were taken with an Andor iXion+ camera, controlled by Andor SOLIS software. By importing the images to a Macintosh computer running Adobe Photoshop and NIH ImageJ software, a stack was generated and used to count all of the nuclei in the segment. The counting of nuclei was performed by evaluating all the images in each stack.

Control and experimental fibers were also investigated ex vivo after maceration similar to previous studies (15, 71, 72). After taking sections, the remaining part of the muscles was thawed in 4% paraformaldehyde diluted in relaxing solution (137 mM NaCl, 5.4 mM KCl, 5 mM $MgCl_2$, 4 mM EGTA, 5 mM HEPES, pH 7). The muscles were subsequently incubated in a 40% NaOH solution for 3 h at room temperature followed by 8 min shaking in a 20% NaOH and 3× washing in PBS (pH = 7.4). Watchmaker's forceps and a binocular microscope were used to mount single myofibers on a gelatin-coated glass. For visualizing fibers and nuclei the mounted fibers were stained with hematoxylin and observed through a 60× 0.3 NA objective with a measuring ocular for determining distances.

Statistics. Effects of inactivity on populations of nuclei were tested by ANOVA and Bonferroni's post test for multiple comparisons. Numbers are given as means ± SE unless stated otherwise.

RESULTS

Unloading leads to atrophy but no loss of nuclei. After 14 days of unloading we observed a 34% decrease in CSA of soleus fibers (Fig. 2, *A* and *B*), in agreement with previous studies (47, 67). Muscles from rats caged normally over the same time period displayed no significant changes in CSA (Fig. 2, *A* and *B*).

Myonuclei were identified by applying dystrophin staining to label the fiber cortexes, and determining if each nucleus had its geometric center inside of the dystrophin "ring." Unloading had no effect on the number of such nuclei (Fig. 2*C*), nor did the number change significantly in control animals (Fig. 2*C*).

Since this finding is in opposition with most existing literature, we also investigated myonuclear number in single fibers. First, to establish the in situ nuclear number and sarcomere length of rat soleus fibers within the living animal we injected single fibers in vivo. For technical reasons, injection and imaging were performed with the soleus muscle in the maximally stretched in situ position (foot fully dorsoflexed) resulting in sarcomere lengths ranging from 2.74 to 3.45 μm (average 3.04 μm) (Fig. 3, *A*, *B*, and *E*). At this sarcomere length we found 102 ± 21 nuclei/mm or 0.33 nuclei/sarcomere in vivo [Fig. 3, *C* (top panel) and *D*].

Subsequently, we thawed the remaining part of the muscles that had been sectioned in a relaxing-fix solution and subjected them to maceration. Cross striations were clearly visible in the microscope (Fig. 3*C*) and the control fibers displayed a similar number of nuclei as was observed in vivo, 0.315 ± 0.004

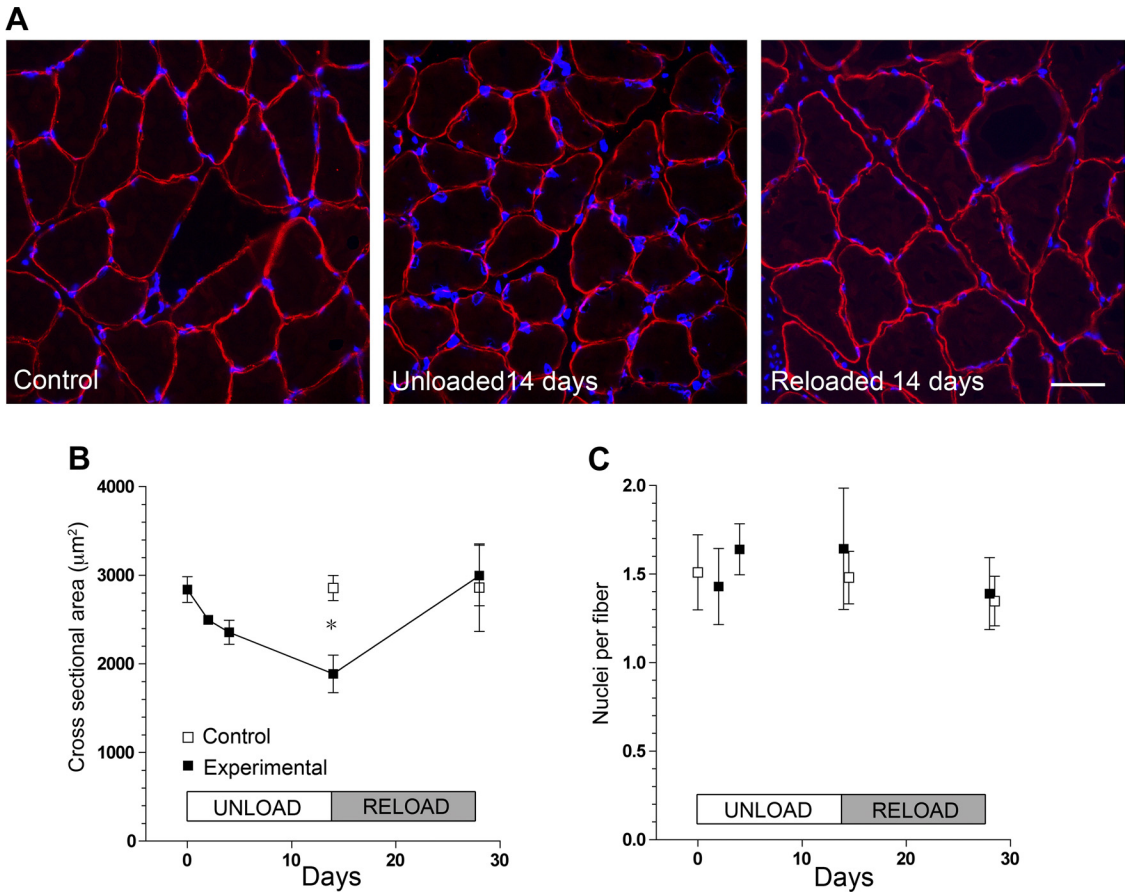

Fig. 2. Effects of unloading on size and number of myonuclei in solei muscles investigated on muscle cryosections. Representative micrographs (*A*) of normal, unloaded and reloaded muscles stained with antibodies against dystrophin (red) and Hoechst 33342 against DNA (blue). Quantification of cross-sectional area (CSA) (*B*) and nuclei per millimeter fiber length (*C*). Each data point represents mean ± SE of 40–92 fibers each from 6–8 muscles. Open symbols represent control muscles and are slightly moved to the right for clarity. Asterisks indicate statistical significance from control values (*$P < 0.05$). Scale bar is 25 μm.

nuclei/sarcomere or 104 ± 18 nuclei/mm when the sarcomere length was standardized to the in vivo value of 3 μm [Fig. 3, *C* (middle panel) and *D*]. When fibers were taken from muscles that had been unloaded for 14 days, the numbers were also not significantly different: 0.324 ± 0.003 nuclei/sarcomere or 108 ± 20 nuclei/mm or 104 ± 18 nuclei/mm when the sarcomere length was standardized to the in vivo value of 3 μm [Fig. 3, *C* (bottom panel) and *D*]. Interestingly the sarcomere length of fibers isolated from unloaded muscles seemed to be more variable, and on average 28% more stretched, than fibers from normal muscles (2.79 ± 0.04 vs. 2.18 ± 0.04 μm; $P = 0.0001$; Fig. 3*E*). Fibers from normal and unloaded muscles with the same sarcomere length displayed the same number of nuclei per millimeter (Fig. 3*F*). In the physiological sarcomere range of approximately 2–3 μm, the correlation between sarcomere length and number of nuclei was fairly linear (Fig. 3*F*).

Unloading leads to apoptosis, but not of myonuclei. In agreement with the observation that there is no loss of myonuclei we found that apoptotic myonuclei were extremely rare in both control and unloaded muscles. Of the more than 200,000 myonuclei that were screened in the present study, only 4 appeared to be TUNEL positive: 0.002% (Fig. 4).

In agreement with previous studies (43) we observed a sevenfold increase in the number of TUNEL-positive nuclei in the muscle tissue after unloading. Thus a midbelly cross section of control muscles showed on average 2 ± 0.4 positive nuclei, while after 14 days of unloading 14.5 ± 2.6 nuclei were displayed (Fig. 4), but virtually all these nuclei had their geometric center outside dystrophin rings.

Reloading leads to relative hypertrophy but no recruitment of myonuclei. During reloading, apoptotic activity in the tissue was strongly reduced but did not reach control levels after 14 days (Fig. 4*B*). Reloading led to a growth of 59% in CSA compared with the *day 14* unloaded levels, and the atrophy was completely restored to preunloading levels (Fig. 2, *A* and *B*). This radial growth was however not accompanied by any significant changes in the number of myonuclei (Fig. 2*C*). We conclude that the number of myonuclei remained unaltered during a complete atrophy-regrowth cycle where the muscle fibers lost and regained approximately 1/3 of their original CSA.

DISCUSSION

The role of myonuclei during atrophy. We have confirmed previous literature that unloading leads to severe atrophy of the slow soleus muscle, accompanied by a pronounced increase in apoptosis in the tissue. However, we find that apoptosis was confined to nuclei outside of the muscle fibers, mainly stroma cells. Myonuclei seem to be protected from apoptosis during unloading. Thus TUNEL-positive myonuclei were exceedingly

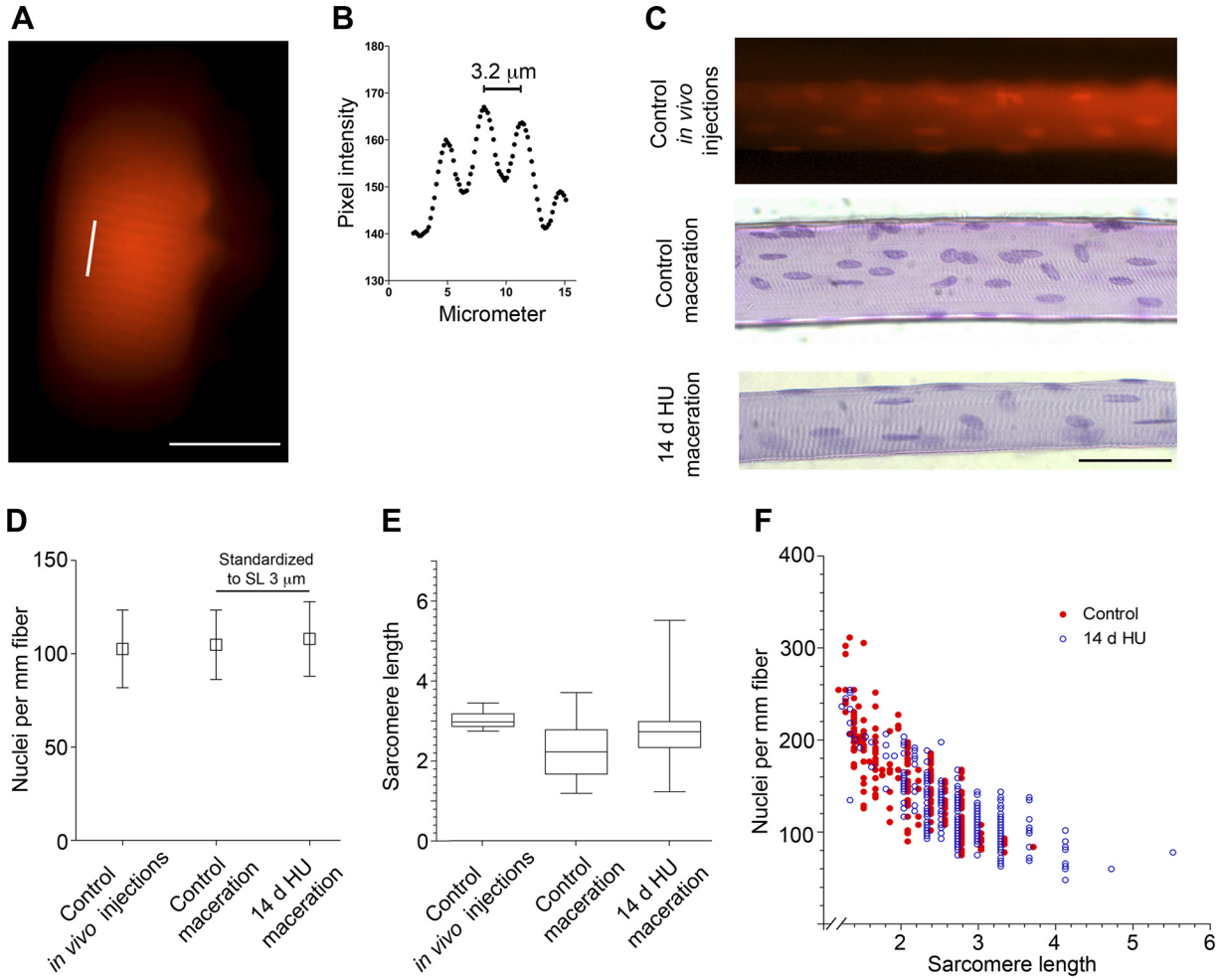

Fig. 3. Effects of unloading on the number of myonuclei in single fibers from soleus muscles. Micrograph illustrating visualization of cross striations seconds after the injection (*A*, scale bar is 30 μm), with intensity measurements of the indicated segment (*B*). Micrographs of single fibers: top panel in vivo; bottom panels ex vivo (*C*, scale bar is 50 μm). Number of myonuclei of single muscle fibers: each data point represents mean ± SE of a total of 23 fibers from 3 muscles in vivo and 234 and 299 from 6 control and 6 unloaded muscles ex vivo (*D*). Sarcomere lengths: the box extends from the 25th to 75th percentiles; the line represent the median, and the whiskers represent maximum and minimum (*E*). Correlation between sarcomere length and number of nuclei/mm fiber. The control data was shifted slightly to the right for clarity (*F*). HU means hindlimb unloaded.

rare, and the number of myonuclei was unchanged. This finding is in agreement with several recent experiments based on direct observation of myonuclei by in vivo imaging in experiments utilizing other atrophy models (14, 15, 34), and one previous report on unloading where myonuclei were identified by confocal 3-D reconstruction of mechanically isolated fiber segments (42). Several other unloading studies based on conventional histology (2, 7, 8, 21, 28, 60, 61), including some where the fiber cortex was delineated by dystrophin labeling (25, 43, 48), reported losses of myonuclei. Even with dystrophin staining, however, the identification of myonuclei on sections is problematic. We have noted that a relatively large number of nuclei appear to lie over the dystrophin rings; thus the criteria for determining if it is inside or outside the ring are critical. In the present and previous studies (14, 15, 34) we have defined myonuclei as the only nuclei with their geometrical center inside the inner rim of the dystrophin ring (Fig. 4). When the same operator evaluated the same sections, but counting all nuclei with their geometrical center either on or within the dystrophin ring, we found that the number of myonuclei increased by 85%. Inclusion of nonmyonuclei might confound both values of nuclear counts, and the cellular identity of nuclei undergoing apoptosis. Unfortunately most previous papers do not report precise inclusion criteria for myonuclei. On sections, the Dupont-Versteegden group (43) report at least 30% higher counts of myonuclei (2.2 nuclei/fiber) than we do, as all our averages were below 1.7 nuclei/fibers. Mitchell and Pavlath (48) studied mice and therefore cannot be compared with the rat literature, at least not quantitatively.

Two studies reported a loss of nuclei based on counting myonuclei in isolated muscle fibers (2, 3). In these papers mechanical isolation rather than maceration was used. A previous systematic comparison concluded that while alkali maceration (such as used in the present study) isolates single myofibers without nonmuscle cells attached, mechanical isolation yielded fibers with nuclei from other cell types still attached (72). When studying the images from Allen et al. (2, 3) the orientation and distribution of the nuclei appear different from myonuclei observed in vivo or after maceration (Fig. 3*C*),

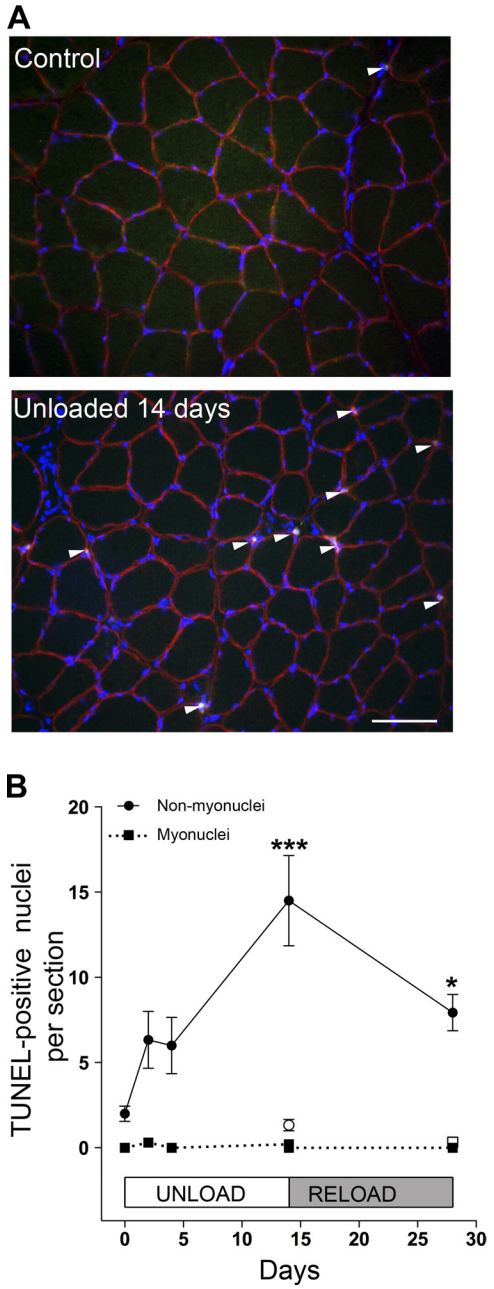

Fig. 4. Apoptosis in normal and unloaded muscle. Micrographs of cryosections stained with TUNEL (green) antibodies against dystrophin (red) and Hoechst 33342 against DNA (blue). Arrowsheads point to the TUNEL-positive nuclei; scale bar is 25 μm. (*A*). Counts of apoptotic nuclei (means ± SE) are based on 2–3 sections from 6–8 muscles. Open symbols represent control muscles. Asterisks indicate statistical significance from control values from muscles before unloading: $*P < 0.05$; $***P < 0.001$ (*B*).

suggesting that some of the nuclei were indeed not myonuclei. This interpretation is supported by the finding that in these papers the myonuclear numbers were as high as 160 and 256 nuclei/mm, respectively, while for living rat soleus fibers in vivo (Fig. 2) and for macerated fibers we find average numbers below 110 nuclei/mm. Kasper and Xun (42), who reported no loss of myonuclei after hindlimb suspension, also studied mechanically isolated fibers, but contamination of nonmuscle nuclei may have been less of a problem since control fibers displayed 128 nuclei/mm at a sarcomere length of 3 μm.

Variability in stretch is another confounding problem. We here report a systematic variability in sarcomere length in ex vivo preparation of single fibers in that unloaded fibers appear more stretched. Mechanistically this might be related to unloading causing a decreased calcium sensitivity of the contractile apparatus (47) (less ex vivo contraction) or to decreased passive contraction caused by a decrease in titin content (68). Allen et al. (2, 3) did not record sarcomere length. We, however, in addition to Kasper and Xun (42), corrected and standardized for the variability in sarcomere length and found no loss of myonuclei.

Since identification of myonuclei on sections can be problematic, this may explain the variable observations with respect to apoptosis in such nuclei. Another possible error source is that TUNEL staining is prone to false positives (29, 53). Thus it is relevant that when myonuclei appeared to become apoptotic in one of the previous reports (25), the frequencies of apoptotic nuclei in control muscles were four times as high as those we report here, and have reported previously (14, 15, 34).

We conclude that direct observation with in vivo imaging and more stringent ex vivo histological methods have demonstrated that nuclei are not lost during hindlimb suspension or other disuse models (this paper and 14, 15, 22, 34, 42, 72) despite considerable apoptosis occurring in other cell types within the tissue. Moreover, based on theoretical considerations it could be questioned if nuclear loss by a selective nuclear apoptosis is at all possible inside the intact syncytium of a muscle fiber (34). Since such apoptosis has been postulated to be important in a variety of disuse conditions and other pathological conditions in human muscles (64, 65), it seems prudent now to reinvestigate the role of myonuclei with conservative histological methods such as those used in the present paper both for identifying myonuclei and apoptosis.

There has been considerable speculation as to why there is an increase in apoptosis during atrophy; and since it was believed that it represented a loss of myonuclei, it has generally been assumed that it was a mechanism to lose "unnecessary" myonuclei that may represent a metabolic burden, or some other disadvantage. Since apoptosis seems to be confined to satellite cells and/or stroma cells, a different teleological explanation has to be sought. The increased frequency of apoptotic nuclei might reflect removal of excess connective tissue or structures related to the shrinkage of tissue volume. Disuse, however, has been reported also to increase mitosis to about the same degree as apoptosis (39), so it might be more a question of increased turnover of cells rather than removal of excess cells.

The role of myonuclei during hypertrophy. Although hypertrophy can be initiated without recruitment of new myonuclei when satellite cells are genetically ablated (46), there seems to be substantial evidence (4, 5, 18–20, 26, 30, 41, 44, 45, 50, 51, 54, 55, 58, 73), including data from in vivo imaging (15), that there is a recruitment of myonuclei under at least some hypertrophy conditions. When a normal satellite cell pool is present the recruitment of myonuclei seems to precede the hypertrophy (15). Since these nuclei seem to be long lasting, they might provide a form of "muscle memory" storing information about previous size, with an increased synthetic capacity facilitating subsequent radial regrowth (15).

We here asked if muscle growth per se is accompanied by myonuclear recruitment. Upon reloading, a 59% increase in

CSA was observed without any increase in the number of nuclei. The relative growth is larger than in some of the hypertrophy experiments where addition of nuclei have been reported, including hypertrophy caused by synergist ablation of the same muscle (soleus) and species (rats) as in the present study (45), and also with in vivo imaging of the fast mouse extensor digitorum longus muscle in mice (15). Since no increase in myonuclei number was observed despite the large growth, we conclude that myonuclear recruitment is not related to relative fiber growth per se. More specifically we suggest that the number of nuclei increases only if a fiber grows beyond a size it has previously had. As a corollary, the number of nuclei would reflect the largest size the fiber has had in its history.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.C.B., D.D., and K.G. conception and design of research; J.C.B., I.M.E., T.K.L., S.D.-A., and D.D. performed experiments; J.C.B., I.M.E., T.K.L., D.D., and K.G. analyzed data; J.C.B., D.D., and K.G. interpreted results of experiments; J.C.B. and K.G. prepared figures; J.C.B., D.D., and K.G. edited and revised manuscript; J.C.B., D.D., and K.G. approved final version of manuscript; K.G. drafted manuscript.

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