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Fiber-Type Transitions and Satellite Cell Activation in Low-Frequency-Stimulated Muscles of Young and Aging Rats

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We examined satellite cell content and the activity of satellite cell progeny in tibialis anterior muscles of young (15 weeks) and aging (101 weeks) Brown Norway (BN) rats, after they were exposed for 50 days to a standardized and highly reproducible regime of chronic low-frequency electrical stimulation. Chronic low-frequency electrical stimulation was successful in inducing fast-to-slow fiber-type transformation, characterized by a 2.3-fold increase in the proportion of IIA fibers and fourfold and sevenfold decreases in the proportion of IID/X and IIB fibers in both young and aging BN rats. These changes were accompanied by a twofold increase in the satellite cell content in both the young and aging groups; satellite cell content reached a level that was significantly higher in the young group ($p < .04$). The total muscle precursor cell content (i.e., satellite cells plus progeny), however, did not differ between groups, because there was a greater number of satellite cell progeny passing through the proliferative and differentiative compartments of the aging group. The resulting 1.5-fold increase in myonuclear content was similar in the young and aging groups. We conclude that satellite cells and satellite cell progeny of aging BN rats possess an unaltered capacity to contribute to the adaptive response.

AGING is accompanied by a loss of functional ability and a restricted adaptive potential. At the level of skeletal muscle fibers, the major changes associated with aging include a decline in mass and strength caused by fiber atrophy, fiber loss, and a shift toward slower phenotypes (for reviews, see 1–3). Furthermore, aging muscle has been shown to display lower adaptive (4) and regenerative capacities (5), caused by decreases in satellite cell content (6) and proliferation potential (7).

Another age-related change, especially seen in muscles abundant in mitochondria, is the appearance of muscle fibers with point and/or deletion mutations of mitochondrial DNA (for reviews, see 8 and 9). At the protein level, these changes may result in defects of mitochondrially encoded proteins such as cytochrome c-oxidase (COX), a multimeric enzyme with three mitochondrially encoded subunits. Thus, COX-deficient fibers are typically found in aging muscle (10,11). In a previous study, we investigated effects of enhanced contractile activity by chronic low-frequency electrical stimulation (CLFS) on the incidence of COX-deficient fibers in muscles of young and aging rats (12). In stimulated young muscle, we observed an increase in COX-deficient fibers concomitant with the increase in mitochondrial content. In aging muscle with a similar increase in mitochondrial content, CLFS, however, led to a decrease in the number of COX-deficient fibers. As indicated by the appearance of some very small COX-positive fibers, CLFS obviously enhanced fiber regeneration in aging muscle, thus decreasing the fraction of COX-deficient fibers.

The formation of new fibers in aging muscle obviously disagrees with findings on a reduced regenerative capacity and decrease in satellite cell content and proliferative potential in old muscle. Our observations, however, are in line with the notion that neuromuscular activity has an impact on satellite cell content. We, therefore, undertook the present study to compare the effects of enhanced neuromuscular activity on satellite cell content and proliferative activity of a fast-twitch muscle in young and aging rats. Because it is impossible to create conditions under which young and aging animals perform voluntary exercise at high and identical intensities, CLFS was chosen as a standardized and reproducible regimen of increased neuromuscular activity. CLFS activates all motor units of the stimulated muscle, even those normally not recruited by exercise training. It imposes higher levels of activity over time than any exercise regimen and, therefore, challenges the adaptive potential of the target muscle to its limits. These attributes make CLFS an ideal experimental model for studying effects of enhanced contractile activity on various structural, functional, metabolic, and molecular properties.

The same experimental animals and experimental conditions were applied as in our preceding studies (12,13): Briefly, the left tibialis anterior (TA) muscles of 15-week-

old and 101-week-old male rats of the genetically pure-bred Brown Norway (BN) strain were exposed to CLFS at 10 Hz, 10 hours daily, for 50 days. The contralateral, unstimulated muscles served as an intra-animal control. The 15-week-old rats were considered to be young adults, whereas the 101-week-old animals were considered to be aging. This assignment was based on the average life span (~120 weeks), which represents the 50% survival rate and has traditionally been regarded as the point at which BN rats are considered old or senescent (14).

Changes in myosin heavy chain (MHC)-based fiber-type distribution and fiber size were assessed immunohistochemically on serial cross sections of the stimulated and contralateral unstimulated muscles. Quiescent satellite cells were identified immunohistochemically (15,16) by using an antibody specific for M-cadherin, a calcium-dependent transmembrane glycoprotein, which anchors satellite cells to the sarcolemma (17). As a way to identify proliferating satellite cell progeny, sections were stained for Ki-67, a nuclear nonhistone protein that is expressed in all phases of the cell cycle, except the quiescent *Go* phase (18,19). Satellite cell progeny, newly fused to adjacent fibers, were identified by their intense immunohistochemical reaction for the muscle-specific transcription factor myogenin, which is strongly upregulated during terminal differentiation (20–22). Intrafiber myonuclei were distinguished from interfiber nuclei by staining for hematoxylin and laminin, whereas changes in capillary density were assessed by laminin staining only (23).

Methods

Animals and Low-Frequency Stimulation

Five young adult and five aging male BN rats (TNO Prevention and Health Center for Ageing Research, Leiden, The Netherlands) were utilized in this study. Young and aging rats were 15 and 101 weeks of age at the beginning and 22 and 108 weeks of age at the end of the stimulation period, respectively. All experiments were approved by the local government (Regierungspräsidium Freiburg, Germany) and rats were treated in accordance with established principles of care and use. Rats were housed in the Animal Research Center of the University of Konstanz in a thermally controlled room maintained at 22°C with 12-hour dark cycles alternated with 12-hour light cycles. All rats received food (Altromin 1314, Altromin GmbH, Lage, Germany) and water ad libitum. Electrodes were implanted laterally to the peroneal nerve of the left hind limb (24). After 1 week of recovery, CLFS was initiated (continuously for 10 hours daily at 10 Hz; impulse width of 0.3 milliseconds) and proceeded for 50 consecutive days. Following completion of the stimulation period, rats were euthanized and the TA muscles of the stimulated (left) and contralateral control (right) legs were excised, weighed, and frozen in melting (−159°C) isopentane. Muscles were stored in liquid nitrogen until they were analyzed.

Antibodies

To examine changes in fiber-type distribution, the following monoclonal antibodies directed against adult MHC isoforms were used: MHCI [NOQ7.5.4D (25)]; MHCIIa [SC-71 (26)], and all fast MHC isoforms with the exception of MHCIIx/d [BF-35 (26)]. Embryonic MHC (MHC_{emb}) was detected on frozen sections by using anti-MHC_{emb} clone 47A (27). Mouse monoclonal anti-Ki-67 (clone MIB-5; 18,19) was obtained from Dianova (Hamburg, Germany). Anti-dystrophin monoclonal antibody (clone DYS2, Dy8/6C5) directed against the carboxyl terminus (28) was obtained from Novocastra Laboratories (Newcastle, UK). Monoclonal anti-desmin (clone DE-B-5) and monoclonal anti-vimentin (clone V9) were obtained from Boehringer Mannheim (Mannheim, Germany) and Sigma (Diesenhofen, Germany), respectively. Goat polyclonal anti-M-cadherin (N-19) and rabbit polyclonal anti-myogenin (M-225) antibodies were obtained from Santa Cruz Biochemicals (Santa Cruz, CA). Rabbit polyclonal anti-laminin (IgG) was obtained from ICN Biochemicals (Costa Mesa, CA).

Biotinylated horse-anti-mouse-IgG (rat-absorbed, affinity-purified), biotinylated goat-anti-rabbit-IgG, and biotinylated horse-anti-goat-IgG were obtained from Vector Laboratories, Inc. (Burlingame, CA). Nonspecific control mouse-IgG, goat-IgG, and rabbit-IgG antibodies were obtained from Santa Cruz Biochemicals.

Immunohistochemistry for Myosin, Ki-67, Dystrophin, Vimentin, and Desmin

Frozen, 12-μm-thick sections of TA muscles were air dried, washed once in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-Tween) and twice in PBS, and then incubated for 30 minutes in 3% H_2O_2 in methanol. Sections were subsequently washed and incubated at room temperature for 1 hour in a blocking solution (BS-1: 1% bovine serum albumin, 10% horse serum, and 0.1% PBS-Tween, pH 7.4). Excess BS-1 was removed and sections were incubated (15 minutes) in Avidin-D blocking solution (Vector Laboratories), rinsed with PBS, incubated (15 minutes) with Biotin blocking solution (Vector Laboratories), and washed with PBS. Primary anti-mouse-IgG monoclonal antibodies were diluted in BS-1 as follows: NOQ7.5.4D, culture supernatant 1:400; SC-71, 3.8 μg/ml; BF-35, 1.7 μg/ml; DYS2, culture supernatant 1:8; V9, 4 μg/ml; DE-B-5, 6 μg/ml. Culture supernatants containing anti-MHC_{emb} (clone 47A) and anti-Ki-67 (clone MIB-5) were applied neat. All primary antibodies were applied for 1 hour at room temperature, except anti-Ki-67, which was applied overnight at 4°C. Control sections were completed in parallel in which (a) the primary antibody was substituted with nonspecific control mouse IgG, and (b) the primary antibody was omitted. Sections were washed as before and reacted for 30 minutes with biotinylated horse-anti-mouse-IgG. Sections were then washed, incubated with Biotin-Avidin-horse-radish peroxidase (HRP) complex for 60 minutes, washed again, and reacted for 5 minutes with a substrate solution containing DAB, H_2O_2, and $NiCl_2$ in 50 mM Tris-HCl, pH 7.5 (Vector Laboratories). Reactions were stopped by washing with distilled water. After dehydration in ethanol, sections were cleared and mounted with Entellan (Merck, Darmstadt, Germany).

Immunohistochemistry for Myogenin, M-cadherin, and Laminin

Sections that were 12 μm thick were air dried and then fixed for 10 minutes in cold acetone (−20°C); excess acetone was allowed to evaporate. Sections were then washed once in PBS-Tween and twice in PBS, incubated for 30 minutes in 3% H_2O_2 in methanol, washed again, and incubated for 1 hour in a blocking solution. BS-1 was used for M-cadherin staining, whereas goat serum was substituted for horse serum in a second blocking solution (BS-2) and used for myogenin and laminin staining. Endogenous Avidin and Biotin binding were blocked as previously de-

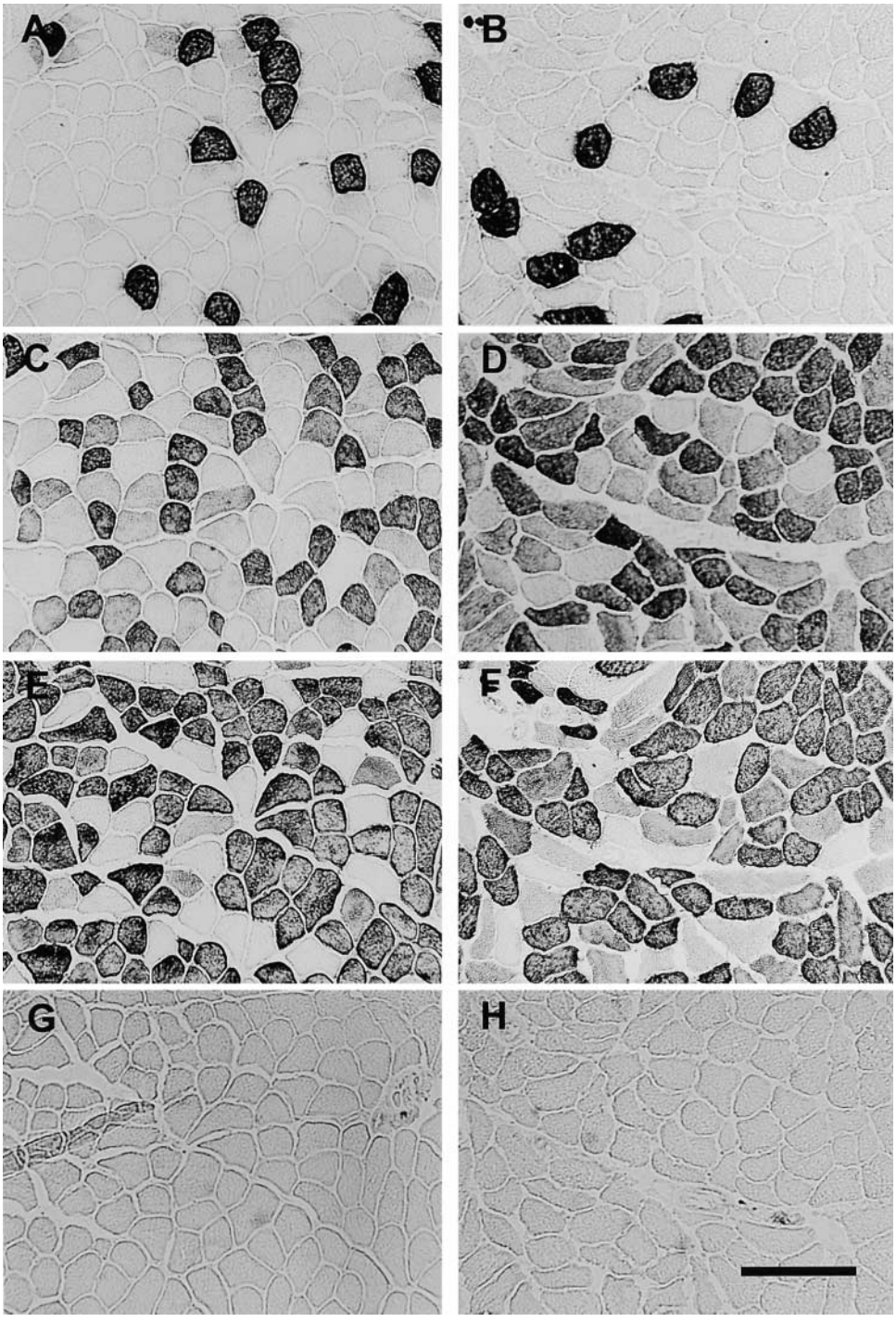

Figure 1. Representative photographs of myosin heavy chain (MHC) immunostains of control (**A**, **C**, **E**, **G**) and 50-day stimulated (**B**, **D**, **F**, **H**) tibialis anterior muscles of an aging rat. **A**, **B**: immunostains for MHCI (clone NOQ7.5.4D); **C**, **D**: immunostains for MHCIIa (clone SC-71); **E**, **F**: immunostains for all MHCs except MHCIId/x (clone BF-35); **G**, **H**: IgG control. Bar is 150 μm.

scribed. Primary polyclonal antibodies were diluted as follows: anti-myogenin to 0.3 μg/ml in BS-2; anti-M-cadherin to 0.7 μg/ml in BS-1; anti-laminin 1:300 in BS-2. Excess blocking solution was removed, and primary antibodies were overlaid on sections and incubated overnight at 4°C. Sections were then washed once in PBS-Tween, washed twice in PBS, and reacted for 30 minutes with either biotinylated goat-anti-rabbit-IgG (for anti-myogenin M-225 and anti-laminin) or horse-anti-goat-IgG (for anti-M-cadherin N19). After washing, sections were incubated for 60 minutes with Biotin-Avidin-HRP, washed, and reacted for 6 minutes with the substrate solution (previously described). Immunolocalization of laminin was visualized with Vector Fast Red (Vector Laboratories). After the reaction was stopped with distilled water, those sections stained for laminin were counterstained with Mayer's Hemalum (Merck) and used to evaluate the total number of muscle nuclei and capillary density. All sections were subsequently dehydrated, cleared, and mounted in Entellan.

Immunohistochemical Analyses

Serial sections, stained for the various MHC isoforms, were examined by using a computer program (28) to determine the relative proportion of muscle fiber types. A similar number of fibers were examined from two distinct areas (superficial and deep) for each of the stimulated (total fibers: 431 ± 28 fibers/muscle) and control legs (total fibers: 380 ± 25 fibers/muscle). A total of 8094 fibers were examined for these analyses: aging control, 1838 fibers; aging stimulated, 2151; young control, 1943; young stimulated, 2162. A minimum of 25 and a maximum number of 1680 fibers were examined to assess differences in fiber cross-sectional area, according to fiber type and experimental condition. Because the number of fibers staining positive for MHC_{emb} was small by comparison, they were not included in these analyses but were evaluated separately over the entire cross-sectional area of each muscle.

Serial sections stained for vimentin, desmin, and dystrophin were examined to determine the number of damaged fibers after 50 days of CLFS (28,29). The criteria for identifying a damaged fiber were as follows: vimentin positive, dystrophin negative, and/or an altered pattern of desmin staining (i.e., absence of staining or foci of positivity); the entire cross section of each muscle was examined. Subsequently, the total number of fibers expressing MHC_{emb} was determined for each muscle. Each of these analyses encompassed an area in excess of 25 mm^2 for each of the 20 muscles studied. The mean cross-sectional areas examined for M-cadherin (i.e., quiescent satellite cell), total nuclei (i.e., Mayer's Hemalum and laminin), Ki-67 (i.e., proliferating satellite cell progeny), and myogenin (i.e., terminally differentiating satellite cell progeny) stains were 25.5 ± 2.5, 5.3 ± 0.4, 19.5 ± 2.2, and 16.3 ± 2.3 mm^2, respectively. Capillaries were identified according to their pattern of laminin staining (23), and the number per unit area was evaluated over the entire cross section of each muscle.

Statistical Analyses

Data are presented as mean ± standard error of the mean and analyzed as follows. Unless otherwise stated, differences between the young and aging groups were assessed by an independent samples Student's *t* test, whereas differences between control and stimulated conditions were assessed by a paired dependent samples Student's *t* test. Because changes in fiber-type distribution and relative satellite cell content were predicted a priori, these data were evaluated with one-tailed tests. Differences between individual fiber cross-sectional areas were detected by a two-way analysis of variance, with repeated measures within animals (i.e., stimulated vs control). When a significant *F* ratio was found, differences were located by using the Newman-Keuls post hoc analysis. Differences were considered significant at $p < .05$.

Results

Fiber-Type Transitions

Fiber-type transitions were assessed immunohistochemically on serial sections (Figure 1), in both the deep and superficial regions of each TA muscle. Because no differences were detected between the two experimental groups in ei-

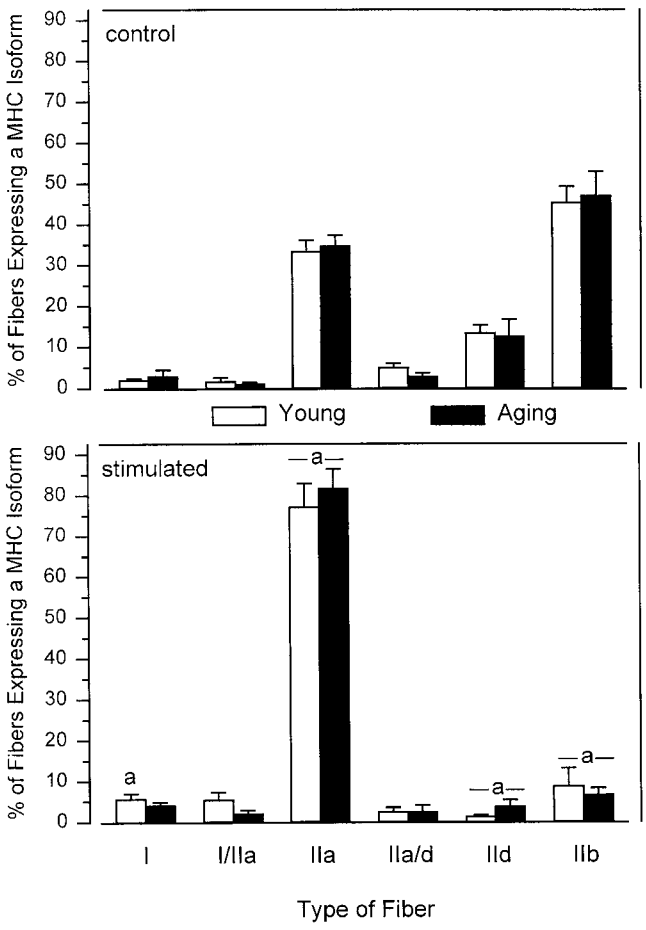

Figure 2. Percentage of distribution of immunohistochemically defined pure and hybrid fiber types in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control; MHC = myosin heavy chain.

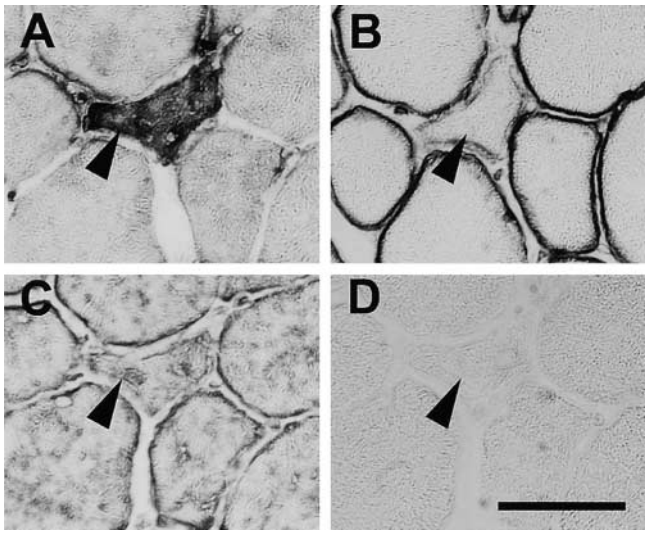

Figure 3. Example of a regenerating fiber that is stained for vimentin (**A**), dystrophin (**B**), desmin (**C**), and IgG control (**D**). Note the presence of vimentin in **A**, the absence of dystrophin (**B**), and a small foci of desmin positivity (**C**) within the fiber marked with an arrow. Bar is 50 μm.

ther of the stimulated or control conditions, the resulting fiber-type distributions of the two regions were averaged. Fifty days of CLFS induced similar fast-to-slow fiber-type transitions in young and aging rats, namely approximately twofold increases in the proportion of IIA fibers and approximately fourfold and sevenfold decreases in the proportions of IID(X) and IIB fibers, respectively (Figure 2). The proportion of fibers expressing MHCI (i.e., type I + type I/IIA) increased by 1.6- and 3.1-fold as a result of CLFS, in the aging and young groups, respectively. Statistical significance was, however, only observed in the young group ($p < .05$), in which the total proportion of MHCI-expressing fibers increased from 3.5% to 10.9% (Figure 2).

The mean cross-sectional area of fibers from contralateral control muscles of the young group was 2034 ± 92 μm^2, and it was slightly larger ($p < .05$) in the aging rats at 2203 ± 576 μm^2. Further examination of the fiber-type-specific cross-sectional areas revealed that the difference was primarily due to significantly larger IIA fibers in the aging group before (young vs aging: 1563 ± 354 vs 1654 ± 406 μm^2; $p < .01$) and after CLFS (young vs aging: 1155 ± 144 vs 1450 ± 320 μm^2; $p < .0001$). CLFS had similar effects on both groups; that is, it reduced ($df = 4822$; $F = 150$; $p < .00001$) the mean cross-sectional areas of the stimulated muscles to 1626 ± 382 μm^2 in the young group and to 1689 ± 411 μm^2 in the aging group. Stimulated fibers remained larger in the muscles of aging rats compared with those in the young stimulated group ($p < .02$).

Structural Morphology, Capillaries

Examination of serial sections for damaged fibers, identified as vimentin positive, dystrophin negative, and/or an altered pattern of desmin staining (Figure 3), revealed no significant differences between young and aging muscles. In the 20 muscles examined, only 25 fibers were detected that met the described criteria. What is more, only 3 were observed in all of the control muscles, whereas the remaining 22 were equally distributed between the stimulated muscles of the young and aging groups.

Examination of the incidence of small myotubes or very small fibers expressing MHC_{emb} revealed 10- and 50-fold elevated ($p < .05$) values in the control and stimulated TA muscles of the aging rats, respectively (Figure 4). Despite the much greater incidence in aging muscles, the absolute numbers of myotubes or small regenerating fibers amounted

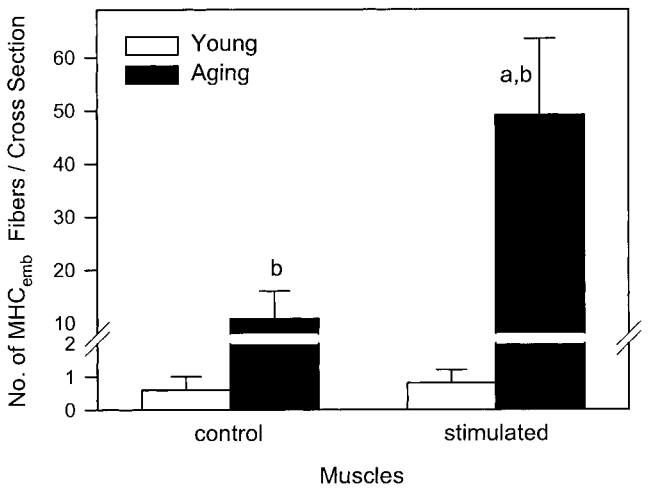

Figure 4. Number of fibers per unit area staining positive for embryonic myosin heavy chain (MHC) in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control; b = aging different from young.

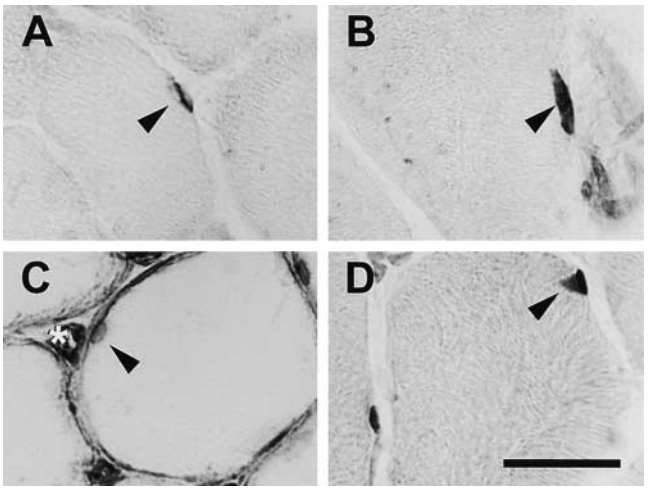

Figure 5. Representative photographs of immunostains of M-cadherin (**A**), Ki-67 (**B**), laminin and hematoxylin (**C**), and myogenin (**D**). These stains were used to identify **A**, satellite cells (arrow); **B**, proliferating satellite cell progeny (arrow); **C**, intrafiber muscle nuclei (arrow) and capillaries (asterisk); and **D**, satellite cell progeny committed to terminal differentiation (arrow). Bar is 25 μm.

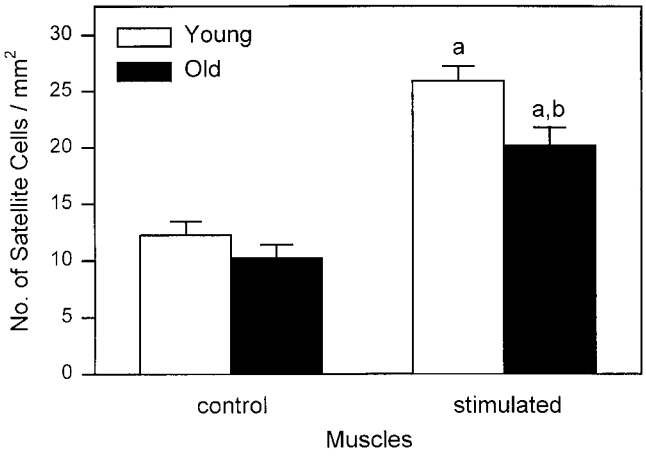

Figure 6. Number of satellite cells per unit area in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control; b = aging different from young.

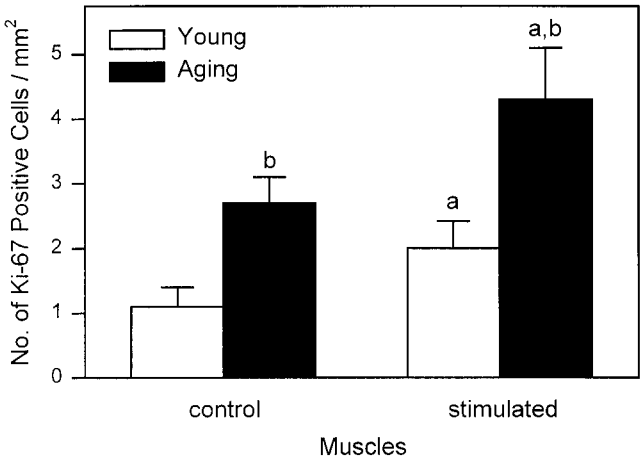

Figure 8. Number of proliferating satellite cell progeny (Ki-67 positive) per unit area in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control; b = aging different from young.

to only 10 and 50 over the entire section, or less than 0.3% and 2% of the muscle fibers present, respectively.

CLFS induced similar 1.8-fold increases in the number of capillaries per unit area in the young and aging groups (Figure 5). In the young group the capillary density increased from 340 ± 5/mm^2 before to 614 ± 26/mm^2 after 50 days of CLFS ($p < .05$). Similarly, in the aging group the number of capillaries per square millimeter increased from 382 ± 30 to 677 ± 62 after CLFS ($p < .05$).

Myonuclear Content and Satellite Cell Activity

The number of satellite cells per unit area, as determined by immunohistochemical patterns of M-cadherin staining (Figure 5), was ~20% greater in control muscles of young rats than in those of aging rats, but it did not reach statistical significance ($p = .32$). Fifty days of CLFS induced more than twofold increases in the satellite cell contents in both groups (Figure 6), reaching a 30% higher level in the young muscles than in the aging muscles ($p < .04$). The absolute increases in satellite cells per unit area for stimulated young and aging muscles were 13 ± 1.6/mm^2 and 10.0 ± 0.7/mm^2, respectively. However, when the total number of muscle precursor cells (mpc) was calculated by addition of satellite cells, proliferating satellite cell progeny, and terminally differentiating satellite cell progeny (Figure 7), there was no apparent difference between the young and aging groups.

Fifty days of CLFS resulted in elevated numbers of proliferating satellite cell progeny (Ki-67 positive) and terminally differentiating satellite cell progeny (myogenin positive), both reaching higher values in the aging than in the young muscles. The number of proliferating satellite cell progeny (Figure 8) was 1.8- and 1.6-fold elevated in the young and aging rats, respectively. Similarly, the number of terminally differentiating satellite cell progeny (Figure 9) increased by twofold and threefold in the muscles of aging and young rats, respectively.

The total number of intrafiber nuclei observed per unit area was similar in the young and aging control muscles (Figure 10) and was elevated 1.5-fold in both groups after 50 days of CLFS. However, the proportion of satellite cells relative to the total number of intrafiber myonuclei was greater ($p < .05$) in young stimulated muscles (4.5 ± 0.3%) compared with the same condition in the aging group (3.4 ± 0.3%). In both groups, the stimulated muscles contained a higher percentage of satellite cells than their respective contralateral controls ($p < .05$), namely 3.1 ± 0.3% in the young and 2.5 ± 0.3% in the aging muscles. In contrast, the proportion of total mpc, relative to the total myonuclear content, was similar between young and aging muscles before and after CLFS (aging, stimulated vs control: 4.9 ± 0.6 vs 3.7 ± 0.6%; young, stimulated vs control: 5.3 ± 0.2 vs 3.7 ± 0.2%).

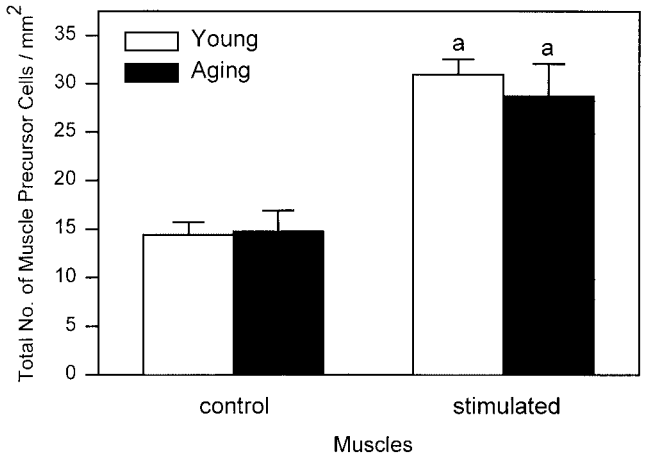

Figure 7. Number of total muscle precursor cells per unit area in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control.

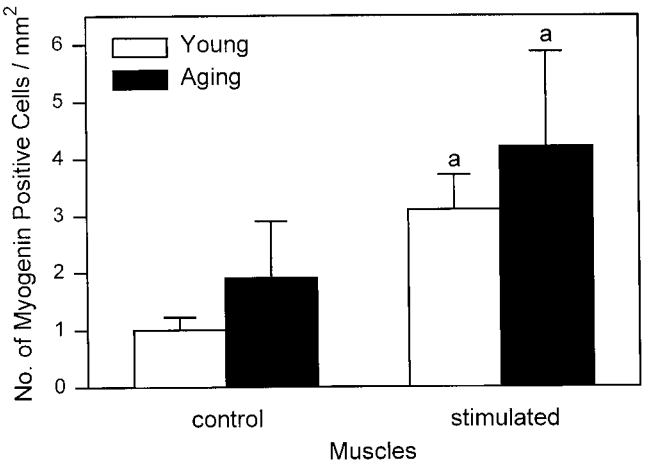

Figure 9. Number of differentiating satellite cell progeny (myogenin positive) per unit area in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control.

Discussion

The present study investigated the effects of 50 days of CLFS on fiber-type transitions and accompanying changes in satellite cell activity in young and aging TA muscles. We investigated aging rather than senescent skeletal muscle because the latter is associated with an intrinsic state of irreversible deterioration (4). In contrast, skeletal muscles of mammals that are late middle-aged to old begin to demonstrate significant functional and structural decline, but they may retain all or part of their adaptive potential (13). Thus we were interested in determining if factors intrinsic to the aging satellite cell population limited the adaptive potential of skeletal muscle or if extrinsic factors associated with an age-related decline in physical activity limited the potential adaptive contributions of satellite cells and their progeny. To that end, a standardized and reproducible model of muscle training was used to examine satellite cell number as well as the potential of aging satellite cells to proliferate and terminally differentiate, in vivo, in response to 50 days of CLFS. This model appeared to be appropriate for two reasons: First, CLFS is known to induce increases in satellite cell activity and content that are temporally and spatially correlated with a large fast-to-slow fiber-type transformation (21,22,28). Second, it has been demonstrated that satellite cells isolated from aging rat skeletal muscle possess significantly lower proliferation potential in culture (7).

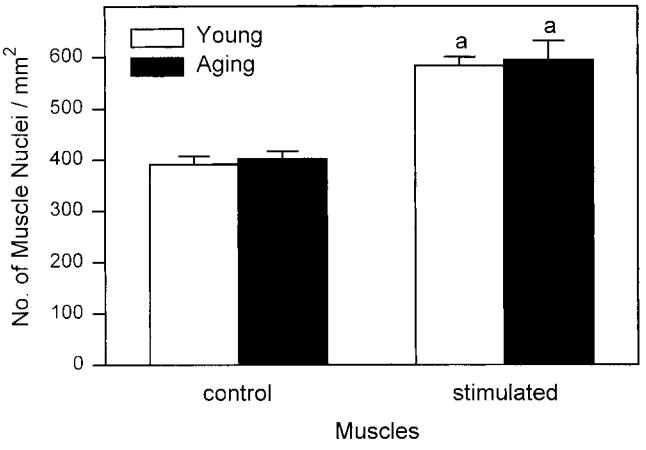

Figure 10. Number of intrafiber muscle nuclei per unit area in control and stimulated tibialis anterior muscles of young and aging rats after 50 days of chronic low-frequency stimulation. a = different from contralateral control.

Age- and CLFS-Related Changes in Fiber Types

With the use of sensitive and highly specific immunohistochemical methods, age-related fast-to-slow phenotypic changes were not detectable in either the deep or superficial regions of control TA muscles of 108-week-old BN rats (Figures 1 and 2). Our results are in agreement with previous studies that used classical histochemical myosin adenosine triphosphate methods to examine age-related phenotypic changes in control muscles of young and old rats (30–33).

Fifty days of CLFS induced fast-to-slow fiber-type transitions in young and aging TA muscles that were quantitatively and qualitatively similar. We interpret this as evidence that aging BN rats possess an adaptive range that is comparable with that of their young counterparts. Both groups demonstrated 2.3-fold increases in the proportion of pure IIA fibers that were in excess of 80% of all fibers examined. Increases in the less fast IIA fibers were primarily offset by reductions in the proportions of the faster pure IID/X and IIB fibers. Differences in the findings of this study and our previous one (13) relate to different analytical methods used and muscles studied. In our previous study (13), quantitative electrophoretic analysis revealed an increased proportion of MHCIId/x and decreased MHCIIb in the control extensor digitorum longus (EDL) muscle of the aging group. This differed from the present study, in which we used a semiquantitative analytical approach and studied the TA muscle, which experiences a lower postural load than the EDL and may therefore be less susceptible to age-related changes in fiber-type profile and MHC complement.

The CLFS-induced phenotypic changes were almost identical in the young and aging groups, but they did differ somewhat with respect to the proportion of fibers expressing MHCI, which was slightly greater in the young group. Despite this small difference, these data clearly demonstrate that CLFS was successful in inducing fiber-type transitions in the fast-to-slow direction. This was especially evident in the superficial region of stimulated TA muscles, where IIA fibers were rare under normal control conditions but were in abundance within the deep region of control and stimulated rat TA muscles. Our inclusion of the superficial region in the present analyses thus explains the larger relative CLFS-induced increase in IIA fibers compared with our previous paper (12), which focused entirely on the deep region of the same TA muscles.

Age- and CLFS-Related Increase in Small Myotubes

The 10-fold increase in the number of small MHC_{emb}-positive myotubes observed in the aging versus young con-

trol muscles (Figure 4) is consistent with the development of an age-related increase in regenerating fibers. Interestingly, CLFS induced a further fivefold increase in the appearance of small myotubes. The reason for the age- and CLFS-related increases in regenerating myotubes is not readily obvious, but it may relate to a mechanical vulnerability of aging muscle (34–36). It is also probable that the increases resulted from neurodegenerative changes (37), leading to denervation and fiber atrophy. These events result in a rearrangement of the fast motor units, increasing their innervation ratios and areas (37). To accommodate for the loss of fibers, the surviving motor units tend to be recruited more frequently, which may facilitate the transformation of the remaining fast fibers toward a slower, more efficient phenotype and/or lead to fiber hypertrophy. The increase in the cross-sectional area of the IIA fibers observed in the present study may be explained by such events.

Satellite Cells

In response to increased contractile activity (22) or chronically increased load bearing (38), satellite cells become activated and leave their quiescent *Go* state. A portion of the new progeny proliferates, proceeds toward terminal differentiation, and fuses to adult fibers or with one another to form new fibers; the remaining progeny reenter the quiescent *Go* phase to replenish the satellite cell pool. Although the exact nature of their long-term contribution(s) to the adaptive response to CLFS remains uncertain, two distinct possibilities exist. First, during the fast-to-slow phenotypic transformation, slow satellite cell lineages are preferentially recruited to transforming fast fibers, where they override the native phenotype. Indeed, Bischoff (39) has reported that exogenously added satellite cells imposed local phenotypic changes on adult fibers in culture. A second and more plausible explanation is that both slow and fast satellite cell lineages are recruited and their phenotype is regulated by local factors released from transforming fibers or their associated nerves. Such plasticity of the satellite cell population has been demonstrated in vitro (40), which suggests that nuclear accreditation is the primary role of satellite cells during phenotypic transformation. In this regard, satellite cells may be considered as important accessories to the transformation process (41).

We have observed that during 20 days of CLFS, transformation in the fast-twitch IIB fiber population follows the time-dependent increase in proliferation and differentiation (21,22) of satellite cell progeny, leading to an increase in myonuclear density (28), which approaches levels typically found in slow-twitch fibers. Most notable was that satellite cell progeny selectively fused to the fastest IIB fiber population in the early phase of adaptation to CLFS (i.e., 5 days), followed by the appearance of MHC_{emb} and a slower MHC isoform (i.e., MHCIIa) in the undamaged transforming IIB fiber population 15 days later (21,22). These findings point to the primary role of satellite cells as providing a source of new myonuclei that is probably both necessary and sufficient for the adaptive response to CLFS.

Whether CLFS-induced fast-to-slow phenotypic transformation is impeded by the absence of a viable population of satellite cells remains unclear. The work of Rosenblatt and colleagues (42,43) suggests that the recruitment of satellite cells may not be necessary for a limited amount of transformation within the subpopulations of fast-twitch fibers. Further examination of this point is, however, required because there are fundamental limitations of the ablation experimental model used (44). Sterilization of the satellite cell pool was not confirmed in those studies. Furthermore, it has been demonstrated that the dose of γ-irradiation used (i.e., 25 Gy) is sufficient to disrupt mitotic activity of the satellite cell pool for only 6 days. The data of those studies were, however, collected 14 days after ablation and irradiation, a full 8 days after the satellite cell pool is known to begin recovery (44). Finally, the magnitude of the stimulus provided by the ablation experimental model used is significantly lower and qualitatively different than that provided by CLFS. The best evidence of this lies in the degree of fast-to-slow transformation, which is much more extensive after CLFS.

We suggest that the primary contribution of the satellite cell population in rat skeletal muscle undergoing CLFS-induced fast-to-slow fiber-type transformation is to provide a necessary and sufficient myonuclear density to first allow and later maintain the transformed state. In the present study the myonuclear density increased by the same absolute (i.e., ∼200 new nuclei/mm^2) and relative (i.e., ∼50%) amounts in both groups, indicating that the satellite cell pools of young and aging BN rats were equally capable of contributing to the myonuclear production during CLFS. Interestingly, the extent of CLFS-induced fiber-type transformation and the relative increase in myonuclear content reported here after 50 days are similar to those observed in our previous experiments after 20 days of CLFS. These observations are consistent with the notion that a threshold myonuclear density is probably achieved within the first 20 days of CLFS, allowing transformation to proceed. Further, the higher myonuclear density may then be sufficient to maintain the newly transformed state.

From previous studies it is apparent that the satellite cell population plays a significant and central role in mediating the adaptive response and maintaining muscle-fiber integrity during growth and adaptation (21,22,28,45,46). It is not clear, however, how much of the developing motor frailty associated with aging results from intrinsic changes to the satellite cell population. Previous studies have reported that the absolute and relative number of satellite cells and the satellite cell proliferation potential decrease in rat fast-twitch muscles with advancing age (6,7). It has also been suggested that these changes may underlie much of the reduction in adaptive capacity of aging skeletal muscle. In the present study, the selection of aging 25-month-old rats was significant because this age is associated with a large reduction in proliferation potential in culture (7) and might be expected to underlie a reduced adaptive potential in vivo. Clearly this did not occur in our study, as the capacities of satellite cell progeny for self-renewal and terminal differentiation were not compromised in our group of aging rats after 50 days of CLFS.

In view of the fact that satellite cells and their progeny may exist in three compartments, we used a panel of anti-

bodies that recognize proteins uniquely expressed in satellite cells and their progeny during the quiescent stage (15), during proliferation (18,19), or during terminal differentiation (20). CLFS resulted in similar increases in the total number of muscle precursor cells present in the young and aging groups; they increased from ~15/mm^2 in the control conditions to ~30/mm^2 after CLFS. Despite displaying similar numbers of total mpc, the young and aging groups differed in the proportion of mpc that was actively cycling through the proliferative and differentiative compartments. Approximately 68% of the mpc population was quiescent in the aging muscle, whereas the remaining 32% were evenly distributed between the proliferative and differentiative compartments. By comparison, the fraction of quiescent mpc was higher in young muscles at 85%, whereas the remaining 15% were actively cycling and also evenly distributed between the proliferative and differentiative compartments. Thus, although CLFS did increase the absolute numbers of quiescent, proliferating, and differentiating mpc in each group, it did not alter their proportions within the three compartments. Whether the same can be said of earlier time points can not be established from the present study. From the results of our previous studies (21,22), however, it is possible to speculate that the satellite cell progeny of the young group may have entered the proliferative and differentiative compartments much earlier in response to CLFS (e.g., 5–10 days), possibly as a result of differences in the availability of biofactors regulating those events. This explanation would also account for the lower proportion of satellite cell progeny actively cycling in our young group after 50 days of CLFS.

The results of the present study indicate that satellite cells of aging BN rats are not limited by intrinsic factors in vivo. It seems plausible, therefore, that differences in the availability of extrinsic factors between young and aging muscle might underlie differences in satellite cell content in our control muscles and others (4,6), as well as possible differences in proliferation potential during exercise training and regeneration (5). Whether the potential difference in extrinsic factors arises secondary to an age-related decline in endocrine, paracrine or autocrine factors remains uncertain. Carlson and Faulkner (47) have demonstrated that, during muscle transplantation, the regenerative potential of skeletal muscle graft is dependent on the age of the host and not the age of the graft and its satellite cell population. Others have noted that decreases in growth factors such as bFGF (48) and IGF-I (49), which regulate mpc proliferation and differentiation, parallel the decline in satellite cell content in old skeletal muscle, whereas restoration of these factors (48,50) in old rats to levels found in young controls can restore the regenerative potential of aging skeletal muscle. Moreover, our recent finding that CLFS enhances the expression of FGF-1 and FGF-2, and their receptors in stimulated TA muscles in vivo and in primary myotubes in vitro (46), is most relevant. Taken together with the results of the present study, this finding suggests that changes in the concentration or availability of extrinsic factors associated with forced contractile activity (i.e., CLFS) may elicit similar responses in aging skeletal muscle satellite cells, if the stimulus is of sufficient intensity and duration.

Conclusions

We examined fiber-type transitions, changes in satellite cell content, and the number of mpc in the proliferative and differentiative compartments after 50 days of CLFS in the fast-twitch TA muscles of young and aging BN rats. Although the extent of the CLFS-induced fast-to-slow fiber type transitions was similar in the young and aging groups, a greater number of small myotubes expressing MHC_{emb} was present in the latter group, which is suggestive of regeneration secondary to age-related changes in α-motor neurons. Fifty days of CLFS induced a significantly greater increase in the number of satellite cells in the young group. However, the total increase in the number of muscle precursor cells present (i.e., satellite cells plus their progeny) did not differ between the young and aging groups, because a greater number of progeny passed through the proliferative and differentiative compartments of the aging group. Thus satellite cells of the aging BN rat retain their capacities to produce progeny and contribute to the adaptive response. Satellite cells of aging skeletal muscles do, however, seem to pass through the proliferative and differentiative compartments more frequently because of new fiber formation. The formation of new myotubes also appears to explain the CLFS-induced reduction in the number of COX-deficient fibers by regenerated small COX-positive type I fibers reported in our preceding study (12). We conclude that satellite cells of aging BN rats are not limited by intrinsic factors but are dependent on extrinsic factors that are determined by the quantity and quality of contractile activity imposed on the muscle. Forced contractile activity thus appears to have a positive effect on aging skeletal muscle by reducing the number and proportion of fibers displaying point mutations of mitochondrial DNA.

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