Denervation effects on myonuclear domain size of rat diaphragm fibers

Bharathi Aravamudan,1 Carlos B. Mantilla,1,2 Wen-Zhi Zhan,1 and Gary C. Sieck1,2

Departments of 1Physiology and Biomedical Engineering, and 2Anesthesiology,
Mayo Clinic College of Medicine, Rochester, Minnesota

Submitted 5 October 2005; accepted in final form 5 January 2006

Aravamudan, Bharathi, Carlos B. Mantilla, Wen-Zhi Zhan, and Gary C. Sieck. Denervation effects on myonuclear domain size of rat diaphragm fibers. J Appl Physiol 100: 1617–1622, 2006. — Denervation (DNV) of rat diaphragm muscle (DIAm) leads to selective atrophy of type IIx and IIb fibers, whereas the cross-sectional area of type I and IIA fibers remains unchanged or slightly hypertrophied. DIAm DNV also increases satellite cell mitotic activity and myonuclear apoptosis. Similar to other skeletal muscles, DIAm fibers are multinucleated, and each myonucleus regulates the gene products in a finite volume of a myonuclear domain (MND). MND size varies across DIAm fiber types in rank order, I < IIA < IIx < IIb [fiber type based on myosin heavy chain isoform expression]. We hypothesized that, after DNV, the total number of myonuclei per fiber does not change and, accordingly, that MND changes proportionately to the change in fiber size regardless of fiber type. Adult rats underwent unilateral (right side) DIAm DNV, and after 2 wk single fibers were dissected. Fiber cross-sectional area, myonuclear number, and MND were measured by confocal microscopy, and these values in DNV DIAm were compared with those obtained in controls. After DNV, type I fibers hypertrophied, type IIA fiber size was unchanged, and type IIx and IIb fibers atrophied compared with control. The total number of myonuclei per fiber was not affected by DNV. Accordingly, after DNV, type I fiber MND increased by 25%, whereas it decreased in type IIx and IIb fibers by 50% and 70%, respectively. These results suggest that MND is not maintained after DNV-induced DIAm fiber hypertrophy or atrophy. These results are interpreted with respect to consequent effects of DNV on myonuclear transcriptional activity and protein turnover.

respiratory muscles; muscle atrophy; skeletal muscle; fiber type; myosin heavy chain

SKELETAL MUSCLE FIBERS ARE multinucleated with each myonucleus controlling the gene products in a finite volume of a muscle fiber-myonuclear domain (MND) (5, 16, 23). The original concept of MND suggested that myoplasmic volume per myonucleus is regulated so that MND is maintained under conditions that induce muscle fiber hypertrophy and atrophy (16, 23). Changes in MND could influence the efficacy of gene transcription with downstream effects on protein synthesis and content. Thus it is important to examine whether MND is maintained under conditions that lead to changes in muscle fiber volume, i.e., hypertrophy or atrophy.

Previous studies have consistently reported that MND remains constant under conditions that lead to muscle fiber hypertrophy (19, 24, 25). However, whether MND is maintained under conditions of muscle fiber atrophy remains controversial. Some studies have reported that MND is maintained during muscle fiber atrophy (2, 17) owing to a proportionate decrease in the number of myonuclei. Other studies have reported a reduction in MND, either as a result of a disproportionate decrease in fiber volume compared with the decrease in the number of myonuclei (2, 4, 36) or as a result of no change in the number of myonuclei despite fiber atrophy (1, 22, 33). Whether a loss of myonuclei is the major contributing factor to muscle fiber atrophy, as concluded by several studies (2, 4, 17, 36), remains the central controversial point.

It is possible that these conflicting results relate to the experimental conditions used to induce muscle fiber atrophy. Most of the previous studies explored changes in MND in hindlimb muscle fibers where atrophy was induced by altered mechanical loading, i.e., hindlimb suspension (4), bed rest (22), spinal cord isolation (1, 36), or spaceflight (2, 17, 26). All of these previous studies examining changes in MND in atrophied muscles focused on hindlimb muscles that are predominantly composed of a single fiber type, e.g., the soleus (1, 2, 4, 17, 19, 22, 36) or extensor digitorum longus muscles (24). It is very likely that differences in fiber-type composition of hindlimb muscles could have influenced these results.

The diaphragm muscle (DIAm), as a major inspiratory muscle, is not influenced by gravity; thus loading conditions on the DIAm differ substantially from those on hindlimb muscles. The DIAm also comprises a mixed fiber-type composition (27, 28) so that all fiber types experience the same experimental condition. In a recent study, we found that MND varies across fiber types in the rat DIAm (33). As in any skeletal muscle, adjusting the balance between synthesis and degradation of contractile proteins is a means by which to affect the size and mechanical properties of DIAm fibers (10–12). After 2 wk of unilateral DIAm denervation (DNV) there was a pronounced atrophy of type IIx and IIB fibers, whereas type I and IIA fibers displayed slight hypertrophy (9, 29, 34, 35). DNV also leads to a sixfold increase in DIAm satellite cell activation after 3 days (14) and to at least a twofold increase in apoptosis in the gastrocnemius muscle (6, 32). Both of these processes can affect the number of myonuclei and, thus, MND. In a previous study, we found that corticosteroid treatment leads to atrophy of type IIx and IIB DIAm fibers similar to that observed with DNV (33). This study also reported that the total number of myonuclei per fiber did not change with atrophy of these fibers and that MND decreased proportionately to fiber size. In contrast, no change in type I and IIA DIAm fiber size was observed with corticosteroid treatment nor was there a change in the number of myonuclei or MND. Given these results we hypothesized that, after DNV, the total number of myonuclei per fiber does not change, and accordingly, MND changes proportionately to the change in fiber size regardless of fiber type.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: G. C. Sieck, 4-184 W. Joseph SMH, Mayo Clinic, 200 First St. SW, Rochester, MN 55905 (e-mail: sieck.gary@mayo.edu).
MATERIALS AND METHODS

Animals. To evaluate the effects of denervation on MND size of DIAm fibers, 10 adult male Sprague-Dawley rats were divided into denervated and age-matched control groups. All animals were housed in separate cages under a 12:12-h light-dark cycle. Purina rat chow and water were provided ad libitum. The Institutional Animal Care and Use Committee at Mayo Clinic approved all experimental procedures.

Phrenicotomy. Unilateral, rather than bilateral, DNV was performed in the adult rats to enhance survival of the animals. Furthermore, a 2-wk period of DNV was selected to match similar procedures performed in previous studies where changes in DIAm size, myosin heavy chain (MHC) protein content, and MHC mRNA expression have been reported (12, 13). Animals were anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) administered intramuscularly. The right phrenic nerve was sectioned in the neck at a point away from the distal nerve stump. In a separate study (results not shown), degeneration of nerve fibers from the distal nerve stump are greatly reduced by 3 days after DNV. The wound was closed with 6-0 Vicryl sutures and treated topically with ointment containing aerosporin, neomycin, and bacitracin. All animals recovered quickly after surgery.

Tissue preparation. After 2 wk, inactivity of the right DIAm was verified by the lack of visible contraction during spontaneous respiration. In previous studies, lack of electromyogram activity was confirmed (20). Animals were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg), and the costal DIAm was rapidly excised. Middorsal DIAm segments were cut into strips, stretched to optimal fiber length (∼1.5 × resting length), and pinned on a piece of cork. Muscle strips were then placed in a 0.1% Triton X-100 relaxing solution for 20 min. Single-fiber electrophoresis was performed in previous studies where changes in DIAm size, myosin heavy chain (MHC) protein content, and MHC mRNA expression have been reported (12, 13). Animals were anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) administered intramuscularly. The right phrenic nerve was sectioned in the neck at a point away from the distal nerve stump. In a separate study (results not shown), degeneration of nerve fibers from the distal nerve stump are greatly reduced by 3 days after DNV. The wound was closed with 6-0 Vicryl sutures and treated topically with ointment containing aerosporin, neomycin, and bacitracin. All animals recovered quickly after surgery.

Single-fiber dissection. As in previous studies (11, 12, 33), DIAm strips were pinned on a Sylgard-coated culture dish containing cooled 100% relaxing solution. From each strip, 20–30 fibers were dissected by using a dissecting microscope (StereoZoom4, Leica Microsystems, Bannockburn, IL). Single fibers were cut into two segments. Aluminum clips were attached to the ends of one fiber segment, which was then placed on a glass slide, fixed in 2% paraformaldehyde for 3 min, and washed in 0.1% PBS. Fibers were then treated for 3 min with 1 mg/ml N-(3-triethylammoniumpropyl)-4-(4-(4-(diethylamino)phenyl)butadienyl)pyridinium dibromide (RH 414; Molecular Probes, Eugene, OR), a membrane-specific dye, followed by a 5-min treatment with 0.2 mM propidium iodide (Molecular Probes) to stain the myonuclei. After the final wash in 0.1% PBS, the fibers were mounted in 100% glycerol (Sigma-Aldrich) and covered with a coverslip. Compression of the fiber by the coverslip was minimized by the use of aluminum clips that served as struts.

Fluorescent labeling. The fiber segment with aluminum clips was carefully placed on a glass slide, fixed in 2% paraformaldehyde for 3 min, and washed in 0.1% PBS. Fibers were then treated for 3 min with 1 mg/ml N-(3-triethylammoniumpropyl)-4-(4-(4-(diethylamino)phenyl)butadienyl)pyridinium dibromide (RH 414; Molecular Probes, Eugene, OR), a membrane-specific dye, followed by a 5-min treatment with 0.2 mM propidium iodide (Molecular Probes) to stain the myonuclei. After the final wash in 0.1% PBS, the fibers were mounted in 100% glycerol (Sigma-Aldrich) and covered with a coverslip. Compression of the fiber by the coverslip was minimized by the use of aluminum clips that served as struts.

Single-fiber imaging and MND size determination. Fibers were imaged on an Olympus Fluoview confocal microscope mounted on a BX51WI microscope (Olympus America, Melville, NY). Fibers were illuminated using a krypton laser and imaged with an Olympus DAPI ×40/1.3-numerical aperture oil immersion objective. A representative single DIAm fiber is shown in Fig. 1. Serial confocal optical sections (step size = 0.5 μm) were obtained by moving the stage in only one direction, thus eliminating any backlash error in the stepper motor. Each optical section was digitized and stored in arrays of 800 × 600 pixels. Pixel dimensions were calibrated using a stage micrometer and were found to be 0.5 × 0.5 μm for the xy-plane (parallel to the microscope stage, by convention). The calculated thickness of optical sections was matched to this dimension, such that each voxel was 0.125 μm³. Optical distortions in the xy- and z-axes were estimated empirically by imaging 10- and 15-μm fluorescently labeled microspheres (FluoSpheres, Molecular Probes). Distortion in the xy-plane was estimated to be <1%; in the z-axis, average distortion was ∼9%. Muscle fiber cross-sectional area (CSA) and volume were estimated on the basis of optical sections obtained at three different positions along a randomly selected 300-μm length of the fiber. The number of myonuclei in each fiber segment was counted, and the average sarcomeric spacing was determined at each of the three positions. These measurements were used to calculate the average CSA of individual fibers and MND size. The total number of myonuclei per

Fig. 1. Representative single diaphragm muscle (DIAm) fiber (type IIx) labeled with RH 414 (shown in green) and propidium iodide (shown in red, with yellow representing dual labeling). Sarcomeric spacing and myonuclei are easily identified. Bar represents 20 μm.
fiber was determined from the average number of myonuclei per micrometer (after adjustment for a sarcomeric length of 2.5 μm) and normalized for a 2-cm fiber. The average fiber volume per myonucleus (MND) was calculated and expressed as micrometers cubed per myonucleus.

Verification of myonuclear staining. To estimate the contribution of satellite cells to the total number of myonuclei, single DIAm fibers were immunohistochemically stained with rabbit antibodies against the satellite cell markers c-met (7) and M-cadherin (18) (Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation in primary antibody (1:1,000), single fibers were washed in 0.1% PBS and incubated in Cy5-conjugated anti-rabbit secondary antibody (1:4,000). After the final wash in PBS, the fibers were labeled with propidium iodide, mounted in glycerol, covered with a coverslip, and imaged by confocal microscopy as described above. Less than 2% of all nuclei were satellite cells (data not shown). Thus the majority of propidium iodide-stained nuclei represent myonuclei at single DIAm fibers, in agreement with previous studies of mechanically isolated muscle fibers (1, 3, 19, 33).

Statistical analysis. Differences in fiber CSA, number of myonuclei per fiber, and MND size across fibers containing MHC isoforms and experimental groups were evaluated by a two-way ANOVA, based on the experimental group and the fiber type. Differences were analyzed post hoc by the Tukey-Kramer honestly significant different test when appropriate. All statistical evaluations were performed with standard statistical software (JMP 5.0.1.2, SAS Institute, Cary, NC). Statistical significance was established at the 0.05 level. Values are means ± SE, unless otherwise specified.

RESULTS

Body weight. All animals used initially weighed 250–280 g. At the time of DIAm harvest, both control and DNV animals still had comparable body weights (300–330 g), in agreement with previous observations that DNV does not cause significant changes in animal body weight (20, 35).

Single DIAm fibers and MHC expression. A total of 318 single DIAm fibers were dissected. At least eight fibers of each type were obtained per animal. Given the limitations associated with the single-fiber dissection technique, it is not possible to estimate reliably the relative distribution of DIAm fiber types in the experimental groups. Consistent with a previous study (12), fibers coexpressing MHC<sub>Slow</sub> and MHC<sub>2A</sub> were dissected more commonly after DNV (n = 37 of 166 fibers) compared with controls (n = 8 of 152 fibers). Fibers coexpressing MHC<sub>Slow</sub> and MHC<sub>2A</sub> were not different in fiber dimensions, myonuclear number, or MND compared with type I fibers and, thus, were omitted from further analyses. Figure 1 shows a representative image of a DIAm fiber.

DIAm fiber dimensions. In agreement with previous studies (10, 11), the CSA of DIAm fibers of control animals varies with fiber type: CSA of type IIx and IIb fibers is considerably higher than that of type I and IIA fibers. In addition, DNV results in fiber-type-specific alterations in CSA (Fig. 2), consistent with studies examining whole DIAm cross sections rather than single DIAm fibers (20, 35). In the present study, type I fibers exhibited a 15% increase in mean CSA (range: 423–1,488 μm<sup>2</sup> in control vs. 742–1,459 μm<sup>2</sup> in DNV; P < 0.05). There was no significant effect of DNV on type IIa fibers (range: 570–1,580 μm<sup>2</sup> in control vs. 329–1,595 μm<sup>2</sup> in DNV; P > 0.05). In contrast, in type IIx and IIb fibers, atrophy was observed: a 46% decrease in mean CSA of type IIx fibers (range: 615–3,001 μm<sup>2</sup> in control vs. 339–1,256 μm<sup>2</sup> in DNV; P < 0.05) and a 67% decrease at type IIb fibers (range: 879–4,444 μm<sup>2</sup> in control vs. 296–1,461 μm<sup>2</sup> in DNV; P < 0.05).

Number of myonuclei. The total number of myonuclei per fiber did not vary across fiber types in either control or DNV groups (Fig. 3). In addition, no fiber-type-specific differences in the number of myonuclei per fiber between the control and DNV groups were evident (range: 967–2,232 myonuclei per fiber in control vs. 632–2,400 in DNV; P > 0.05). Accordingly, there is no correlation between the number of myonuclei per fiber and CSA in either the control (Fig. 4A) or the DNV group (Fig. 4B), indicating that the number of myonuclei does not depend on fiber CSA, irrespective of fiber type.

MND size. In agreement with previous studies (33), MND at type IIx and IIb fibers was significantly larger than that at type I and IIA DIAm fibers in the control group (Fig. 5). DNV resulted in significant fiber-specific differences in MND size. Type I fibers showed a 26% increase in MND size (range: 8,024–21,624 μm<sup>3</sup> in control vs. 8,414–25,404 μm<sup>3</sup> in DNV; P < 0.05). DNV did not change the MND size at type IIx fibers (range: 6,204–19,581 μm<sup>3</sup> in control vs. 6,895–21,467 μm<sup>3</sup> in DNV; P > 0.05). Type IIb fibers (range: 8,245–31,501 μm<sup>3</sup> in control vs. 4,825–15,401 μm<sup>3</sup> in DNV;
has been suggested that MND regulates the amount of mRNA available for protein synthesis, and, as such, is an important determinant of fiber growth or atrophy (16, 19, 25). However, this assumes that mRNA transcription rate remains constant per myonucleus. With this assumption, the amount of mRNA available for protein synthesis would correlate inversely with MND. Assuming no change in mRNA transcription rate, the observed changes in MND of DIAm fibers would suggest that the local concentration of type I (MHC<sub>Slow</sub>) mRNA would be lower and the concentrations of type IIx and IIb (MHC<sub>2X</sub> and MHC<sub>2B</sub>) mRNA would be greater after DNV. However, in a previous study (13), we found that 2 wk after DNV, there was a ~75% reduction in MHC<sub>Slow</sub> and MHC<sub>2X</sub> mRNA levels in the rat DIAm, whereas MHC<sub>2A</sub> and MHC<sub>2B</sub> mRNA decreased only slightly. Thus it is unlikely that MND is a major determinant of DIAm fiber atrophy after DNV.

**Changes in the number of myonuclei.** The number of myonuclei may increase or decrease as a result of satellite cell activation or apoptosis, respectively. Unilateral DNV of the rat DIAm activates satellite cells (14), which may fuse to existing myofibers. This should result in an increase in the number of myonuclei and a decrease in MND size if any net gain of myonuclei occurs. In contrast, in the rat gastrocnemius muscle, myonuclear apoptosis has also been shown to occur after DNV (8), and, if present in the DNV DIAm, this would have resulted in a decrease in the number of myonuclei and an increase in MND. However, in the present study, DNV did not result in an increase or decrease in the total number of myonuclei per DIAm fiber in any fiber type (Fig. 3). It is possible that satellite cell activation and apoptosis occur concurrently, thus resulting in no net gain or loss of myonuclei. This possibility cannot be ruled out by the results of the present study.

**Changes in myonuclear domain with atrophy.** The original concept of MND suggested that myoplasmic volume per myonucleus is a controlled variable, strictly regulated so that it does not change with conditions that induce muscle remodeling and adaptation (16, 23). In hindlimb muscles, MND was shown to remain constant under conditions that lead to muscle fiber hypertrophy. For example, after functional overload via re-
moval of synergistic muscles, MND did not change at the rat soleus (19), rat (25) and cat (1) plantaris, and rat extensor digitorum longus muscles (24). Similarly, MND did not change after muscle fiber hypertrophy induced by IGF-1 treatment (19) or treadmill exercise (25). However, the results of studies reporting changes in MND under conditions leading to muscle fiber atrophy are more equivocal. Some studies have suggested that MND is maintained under conditions of muscle fiber atrophy. For example, Hikida and colleagues (17) reported that atrophy of rat soleus muscle fibers induced by 10 days of spaceflight was associated with no change in MND due to a proportionate decrease in the number of myonuclei. Allen and colleagues (2) also reported no change in MND with atrophy of type II fibers in the rat soleus muscle induced by 14 days of spaceflight. Both of these studies concluded that the loss of myonuclei in rat hindlimb during spaceflight is a contributing factor to the atrophy of muscle fibers.

In contrast to those studies reporting no change in MND with muscle fiber atrophy, other studies have reported a decrease in MND. For example, the atrophy of rat soleus muscle fibers induced by 2 mo of spinal isolation led to a 25% decrease in the total number of myonuclei and a 66% decrease in fiber CSA, thus an overall decrease in MND (36). The atrophy of type I soleus muscle fibers caused by 2 wk of spaceflight was associated with a decrease in MND due to a 16% decrease in the total number of myonuclei and a 42% decrease in fiber CSA (2). In a more complex experimental design, Allen and colleagues (4) reported a decrease in MND in soleus fibers after 2 wk of hindlimb unloading that was preceded by a 7-day period of functional overload induced by ablation of synergistic muscles. In this study, the decrease in MND was associated with a 17% decrease in the total number of myonuclei and a 55% decrease in fiber CSA. However, not all studies reporting a decrease in MND have observed a change in the total number of myonuclei. For example, Allen and colleagues (1) reported that 4 mo of spinal isolation in cats caused atrophy of soleus muscle fibers, but in this case the total number of myonuclei did not change and MND decreased proportionately to the decrease in fiber size. Similarly, a proportionate decrease in MND and soleus muscle fiber size was observed after 4 mo of bed rest in humans (22). In the rat DIAm, the decrease in MND with atrophy induced by DNV was similar to that associated with corticosteroid-induced DIAm fiber atrophy (33). Indeed, similar to DNV, corticosteroid treatment also induced atrophy of type IIx and IIb DIAm fibers, and in both cases there were no changes in the number of myonuclei per fiber.

The apparent equivocal nature of all these previous studies reporting either a decrease or no change in MND during muscle fiber atrophy may reflect differences in muscle fiber type, loading conditions, and/or species. However, it is clear from these studies that changes in MND or the number of myonuclei are not always required for muscle fiber atrophy.

**MHC expression.** Muscle fiber contractile properties depend on MHC protein expression. For example, maximum specific force and velocity of shortening of different fiber types in the rat DIAm vary on the basis of the expression of different MHC isoforms (11, 30, 31). Unilateral DNV causes a decrease in MHC protein content that is most prominent in type IIx and IIb DIAm fibers and leads to a reduction in maximum specific force (12). A decrease in MHC protein content may reflect either a decrease in protein synthesis and/or an increase in protein degradation. Reduced availability of MHC mRNA may underlie a decrease in protein synthesis. In a previous study, we found that after 2 wk of DNV, MHCslow and MHC2X mRNA levels in the rat DIAm decrease, with no detectable change in MHC2A and MHC2B mRNA levels (13). In skeletal muscle fibers, a reduction in MHC mRNA concentration may reflect a decrease in transcription rate or an increase in MND size. In the present study, we found that MND of type IIx and IIb fibers decreased; thus, if transcription rate remained unchanged,
MHC2X and MHC2B mRNA levels should have increased. The fact that mRNA levels for these MHC isoforms decreased (MHC2X) or remained unchanged (MHC2B) after DNV clearly suggests that transcription rate must have decreased. Even though transcription rates were not measured directly in the present study, its results, when taken together with those of Geiger et al. (13), strongly suggest that changes in the total number of myonuclei do not contribute significantly to the DNV-induced atrophy of DIAM fibers.

In conclusion, the present study demonstrates that DNV causes proportionate changes in fiber CSA and MND without changing the number of myonuclei in DIAM fibers. On the basis of available evidence, DNV-induced changes in MHC protein content are not directly related to changes in the total number of myonuclei but more likely result from a complex combination of changes in RNA transcription, protein translation, and posttranslational degradation.

ACKNOWLEDGMENTS

The authors thank Thomas Keller for technical assistance.

GRANTS

This research was supported by National Institutes of Health Grants HL-37680 and AR-51173 and by the Mayo Foundation.

REFERENCES


