Effect of denervation on ATP consumption rate of diaphragm muscle fibers

Gary C. Sieck, Wen-Zhi Zhan, Young-Soo Han, and Y. S. Prakash

Department of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota

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Sieck GC, Zhan W-Z, Han Y-S, Prakash YS. Effect of denervation on ATP consumption rate of diaphragm muscle fibers. J Appl Physiol 103: 858–866, 2007. First published June 7, 2007; doi:10.1152/japplphysiol.00988.2006.—Denervation (DNV) of rat diaphragm muscle (DIAm) decreases myosin heavy chain (MHC) content in fibers expressing MHCslow isoform but not in fibers expressing MHC2X and MHC2A. Since MHC is the site of ATP hydrolysis during muscle contraction, we hypothesized that ATP consumption rate during maximum isometric activation (ATPiso) is reduced following unilateral DIAm DNV and that this effect is most pronounced in fibers expressing MHC2X. In single-type-identified, permeabilized DIAm fibers, ATPiso was measured using NADH-linked fluorometry. The maximum velocity of the actomyosin ATPase reaction (V_{max ATPase}) was determined using quantitative histochemistry. The effect of DNV on maximum unloaded shortening velocity (V_{o}) and cross-bridge cycling rate [estimated from the rate constant for force redevelopment (k_{fr}) following quick release and restretch] was also examined. Two weeks after DNV, ATPiso was significantly reduced in fibers expressing MHC2X, but unaffected in fibers expressing MHCslow and MHC2A. This effect of DNV on fibers expressing MHC2X persisted even after normalization for DNV-induced reduction in MHC content. With DNV, V_{o} and k_{fr} were slowed in fibers expressing MHC2X, consistent with the effect on ATPiso. The difference between V_{max ATPase} and ATPiso reflects reserve capacity for ATP consumption, which was reduced across all fibers following DNV; however, this effect was most pronounced in fibers expressing MHC2X. DNV-induced reductions in ATPiso and V_{max ATPase} of fibers expressing MHC2X reflect the underlying decrease in MHC content, while reduction in ATPiso also reflects a slowing of cross-bridge cycling rate.

The rate of ATP consumption during maximum isometric activation (ATPiso) has been measured in single permeabilized muscle fibers using a NADH-linked fluorometric technique. Several studies have reported that there are intrinsic differences in ATPiso in muscle fibers expressing different MHC isoforms, with fibers expressing MHCslow alone or together, with MHC2B having greater ATPiso compared with fibers comprising MHCslow and MHC2A isoforms (3, 18, 19, 39, 42, 49). These differences in ATPiso are due in part to differences in fiber MHC content (b·n from Eq. 1), but, even when normalized for MHC content, ATPiso of fibers expressing MHCslow is lower than that of fibers expressing fast MHC isoforms (18). During maximum calcium activation, the α_{fs} is comparable across all fiber types, but there are differences in maximum shortening velocity (reflecting cross-bridge cycling rate and g_{app}) that contribute to the fiber-type differences in ATPiso (18).

The maximum velocity of the actomyosin ATPase reaction (V_{max ATPase}) in muscle fibers, as measured by quantitative histochemistry, establishes the upper limit for ATP consumption during work performance for each fiber type in skeletal muscle. The V_{max ATPase} of diaphragm muscle (DIAm) is higher for DIAm fibers expressing MHC2X alone or together with MHC2B compared with MHCslow and MHC2A (39, 46). Across all fibers, ATPiso is substantially less than V_{max ATPase}, which is expected, since ATP consumption rate increases during shortening and work performance in muscle (Fenn effect) (8, 9). The difference between V_{max ATPase} and ATPiso reflects the reserve capacity for ATP consumption in muscle fibers and is higher for fibers expressing MHCslow and MHC2A compared with fibers expressing MHC2X alone or together with MHC2B (39, 43).

In a previous study (12), our laboratory found that, following 2 wk of unilateral denervation (DNV) of the DIAm, MHC content and maximum specific force were dramatically reduced for DIAm fibers expressing MHC2X (it should be noted that MHC2B expression essentially disappeared after DNV) compared with fibers expressing MHCslow and MHC2A isoforms. Following DNV, there was also a decrease in maximum unloaded shortening velocity (V_{o}) of DIAm strips (29, 45, 58) that may reflect either an increase in the relative contribution of type I (MHCslow) and IIa (MHCslow) fibers due to atrophy of IIx and/or IIb (MHC2X and/or MHC2B), or a slowing of cross-bridge cycling rate (g_{app}) of individual fibers. In addition, DNV leads to a decrease in V_{max ATPase} that is more pronounced in DIAm fibers expressing MHC2X (29, 58), which could also reflect either an increase in the relative contribution of type I and IIa (MHCslow) fibers or a slowing of single-fiber cross-bridge cycling rate. In the present study, we hypothesized that unilateral DIAm DNV results in a slowing

Address for reprint requests and other correspondence: G. C. Sieck, Dept. of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905 (e-mail: sieck.gary@mayo.edu).
cross-bridge cycling rate and ATP$_{iso}$ that is more pronounced in fibers expressing MHC2C.

METHODS

Adult male Sprague-Dawley rats (initial body weight ~300 g) were randomly assigned to either control (CTL; $n = 6$) or DNV ($n = 7$) groups. The animals were housed in separate cages under a 12:12-h light-dark cycle, fed Purina Rat Chow, and provided water ad libitum. Body weights were monitored every 3 days during the experimental period. Surgical procedures were performed under aseptic conditions, and recovery of animals from surgery was carefully monitored. All procedures were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

Unilateral DIAm DNV. The procedures for unilateral DNV of the rat DIAm have been previously described in detail (16, 29, 58). Briefly, animals were anesthetized by intramuscular injection of ketamine (60 mg/kg) and xylazine (2.5 mg/kg). After a midline incision, the right phrenic nerve was exposed beneath the sternomastoid muscle. The phrenic nerve was then transected, and an ~2 cm length from the distal end was removed to prevent DIAm reinnervation and to minimize any residual neurotrophic effects emanating from the remaining nerve stump, which we estimated would persist for 1–2 days based on the rate of axoplasmic transport. The wound was closed with 6-0 silk sutures, and the surgical wounds were treated topically with Neosporin ointment (containing neomycin, neomycin, and bacitracin). Typically, animals recovered from the anesthetic within 1 h. In a previous study, our laboratory demonstrated that arterial blood-gas levels were normal in animals after DNV, indicating that ventilation is not compromised by unilateral DIAm paralysis (29).

In the present study, the effects of unilateral DNV were assessed after 2 wk. This time period was chosen based on previous studies demonstrating an effect of DNV on DIAm fiber size, on MHC content in single DIAm fibers, on DIAm force, and velocity of shortening (10, 12, 29, 58, 59).

Measurement of $V_{\text{max}}$ ATPase in single fibers. The quantitative histochemical procedure for measuring the $V_{\text{max}}$ ATPase in type-identified muscle fibers has been previously described in detail (2, 43, 46, 58). Briefly, segments from the right midcostal DIAm were stretched to optimal length (1.5 times resting excised muscle length) (32) before being rapidly frozen in isopentane cooled by liquid nitrogen. The muscle segments were mounted, and serial transverse sections were cut at 10-µm thickness using a cryostat kept at −20°C (model 2800E Frigocut, Reichert-Jung).

To determine $V_{\text{max}}$ ATPase in single muscle fibers, the reaction product of the actomyosin ATPase reaction (amount of P, liberated by ATP) was measured. First, free P$_i$ in the muscle fiber sections was converted to a lead sulfide precipitate by reaction with sodium sulfide. The lead phosphate precipitate was then reacted with a lead ammonium citrate/acetate complex to form a lead sulfide precipitate. The concentration of the lead sulfide precipitate in muscle fiber sections was then determined by densitometry using the Lambert-Beer equation:

$$[\text{NBT-dfz}] = \text{OD} / k \cdot d$$

(2)

where [NBT-dfz] is the nitroblue tetrazolium-disformazan concentration; OD is the optical density of the muscle fiber measured at 550 nm (the peak absorbency wavelength for the lead sulfide precipitate); $k$ is the molar extinction coefficient for the lead sulfide precipitate (1,450 mol$^{-1}$·cm$^{-1}$); and $d$ is the path length for light absorbency (10-µm section thickness).

In previous studies, our laboratory demonstrated that the actomyosin ATPase reaction is not contaminated by other ATPases (e.g., those associated with the sarcolemma and sarcoplasmic reticulum) and that the reaction is linear for at least 9 min (2, 46). Accordingly, a single end-point reaction time of 4 min was selected to limit OD measurements to <1.0 OD unit and thereby minimize measurement error in the image processing system (see below). To determine $V_{\text{max}}$ ATPase, ATP concentration in the incubation media was varied from 0.0 to 5.0 mM, and four replicate OD measurements (in four separate sections) of free P$_i$ were obtained for each single fiber at 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mM ATP (a total of 28 serial sections) at 22°C. This was necessary since sufficient ATP could not be added to the incubation medium to avoid substrate limiting the actomyosin ATPase reaction. A Lineweaver-Burk transformation relating velocity of the actomyosin ATPase reaction (OD$_{550}$/min) and ATP concentration was performed to determine $V_{\text{max}}$ ATPase and the apparent Michaelis-Menten rate constant of the actomyosin ATPase reaction (2, 46).

Based on the stoichiometry of P$_i$ sulfide exchange in the histochemical reaction, $V_{\text{max}}$ ATPase was expressed as millimoles of P$_i$ per liter per minute. However, this unit was converted to nanomoles per millimeter cubed per second to be compatible with that used in the ATP$_{iso}$ (19).

For densitometric measurements, microscopic images of the same region of the muscle in alternate serial sections were digitized at 8-bit resolution (256 gray levels) into a $1,024 \times 1,024$ array of picture elements (pixels) using a video image-processing system (Meta morph). The imaging system was calibrated for densitometry using a set of neutral density filters ranging from 0.02 to 2.00 OD units. In a previous study, our laboratory determined that, for OD values ≤1.0, measurement errors were <1.5% (32). Before digitizing images of the muscle sections, light intensity of the microscope was adjusted to optimize the full use of the 256 gray level range of the video camera but to avoid light saturation. Subsequently, light intensity of the microscope was not adjusted.

Determination of MHC isoform expression in single fibers by immunohistochemistry. MHC immunohistochemistry was used to determine MHC isoform expression in those single fibers in which $V_{\text{max}}$ ATPase was measured. The methods employed for detecting MHC expression (or coexpression) based on immunohistochemistry have been previously described in detail (33). Briefly, alternate serial sections of the same muscle region as those used for measuring the actomyosin ATPase reaction were reacted with mouse primary antibodies against different MHC isoforms. Pairs of mouse IgG or IgM primary antibodies were used, e.g., anti-MHCslow (Novocastra, IgG; 1:100), anti-MHC2A (Blau A4.74, IgG; 1:1), anti-MHC2B (BFF3, IgM, purified from mouse hybridoma, German Collection of Microorganisms and Cell Culture; 1:1) and anti-MHC$_{neo}$ (Novocastra; 1:10). To determine MHC2C$_{iso}$ expression, only a single antibody was used, i.e., anti-MHC2C$_{iso}$ (BF-35, IgG, purified from mouse hybridoma, German Collection of Microorganisms and Cell Culture; 1:1). The primary antibodies were diluted in PBS containing 0.5% bovine serum albumin (5 mg/ml), and muscle sections were incubated in these primary antibodies for ~2 h at room temperature. The sections were then washed in PBS and reacted with Cy3- and Cy5-conjugated secondary antibodies (goat anti-mouse IgG or goat anti-mouse IgM; 1:200) for 3–4 h at room temperature. To assess the specificity of the reactivity against primary antibodies, alternate sections were incubated with only the secondary antibodies. The muscle sections were imaged with an Olympus Fluoview confocal microscope. The imaging system was calibrated for morphometry using a stage micrometer.

Single-fiber preparation. Two weeks after DNV, the rats were reanesthetized, and the right side of the DIAm was rapidly excised. Muscle fiber bundles were stretched to ~1.5 times resting excised muscle length, pinned on cork, and placed in a relaxing solution at 5°C for 24 h consisting of 85 mM K$_2$Cl, 1 mM free Mg$^{2+}$, 5 mM MgATP, 7 mM MgATP, 7 mM EGTA, propionate as the major anion, 10 mmol/L Ca$^{2+}$ (pCa 9), 15 mM creatine phosphate, and 1 mg/ml creatine phosphokinase; imidazole was used to maintain the pH at 7.00 ± 0.02 and to adjust the ionic strength to 150 mM. The fiber bundles were then transferred to relaxing solution containing 50% glycerol (vol/vol) and stored immediately at −20°C for 2–3 wk (5).

Before single-fiber dissection, a fiber bundle was placed in relaxation solution containing 10 mM DTT and dissected under a dissecting microscope. The dissected single fibers were then transferred to
relaxing solution containing 10 mM DTT and 1% Triton X-100 to permeabilize the plasma membrane for 20 min. The permeabilized single fibers were again transferred to 50% glycerol relaxing solution before measurements of muscle contractile properties and ATP consumption rate. These measurements were performed at 15°C.

Measurement of ATPiso. ATPiso was measured using an NADH-linked fluorescence technique (17, 19, 31, 39). The ATP solutions contained the same composition as the relaxing and activating solutions without creatine phosphate and creatine phosphokinase, plus 5 mM phospho(enol)pyruvate (PEP), 0.2 mM reduced NADH, 100 U/ml pyruvate kinase (PK), and 140 U/ml lactate dehydrogenase (LDH). The NADH-linked enzymatic assay involves the following reaction:

\[
\text{ATPase} \\
\text{ATP} \rightarrow \text{ADP} + P_i \\
\text{PK} \\
\text{ADP} + \text{PEP} \rightarrow \text{pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} \rightarrow \text{ladate} + \text{NAD}^+ \\
\text{(fluorescent)} \\
\text{LDH} \\
\text{(nonfluorescent)}
\]

In these reactions, the ATP hydrolyzed by actomyosin ATPase (Eq. 3) is regenerated by the biochemical reaction of ADP and PEP, which is catalyzed by PK (Eq. 4). This reaction is coupled to the reduction of pyruvate to lactate, which is catalyzed LDH and the oxidation of NADH to NAD\(^+\) (Eq. 5). For each mole of ATP regenerated by these coupled reactions, 1 mol of NADH is oxidized to NAD\(^+\). Most important in the quantification of ATP consumption is the fact that NADH is fluorescent, while NAD\(^+\) is nonfluorescent. Thus the rate of decrease in NADH fluorescence signal at 450 nm is proportional to the rate of ATP consumption (actomyosin ATPase activity). The reactions are optimized so that PK and LDH activities are not rate limiting, and there are no competing reactions for ADP.

Single, Triton X permeabilized fibers were mounted between force and displacement transducers in a quartz cuvette that was perfused with solutions containing a free ionized Ca\(^{2+}\) of either 1 nM (relaxing state) or 100 μM (maximal Ca\(^{2+}\) activation). For adjusting optimum sarcomere length and measuring a diameter of single fibers in this system, a charge-coupled device video camera system (COHU, solid-state camera) was used. Using an image-processing system digitized a video image of the single fiber. Sarcomere length was adjusted to 2.5 μm, and fiber diameter was measured at three points along the length of the fiber. In addition, fiber depth (Z-axis diameter) was also estimated. Based on these measurements, fiber cross-sectional areas were calculated. Flow of solution through the cuvette was flushed every 15 s, providing fresh constituents necessary to couple ATP hydrolysis to NADH consumption. The rate of decline in NADH fluorescence during a 15-s period was measured and used to calculate ATP consumption rate based on a prior calibration of the system at known NADH concentrations.

**Reserve capacity for ATP consumption of DIAm fibers.** The difference between V\(_{\text{max}}\) ATPase and ATP\(_{\text{iso}}\) reflects the reserve capacity for ATP consumption. The reserve capacity was calculated as \[1 - (\text{ratio of ATP}_{\text{iso}} \text{ to } V_{\text{max}} \text{ ATPase})\], based on fiber types. Since V\(_{\text{max}}\) ATPase and ATP\(_{\text{iso}}\) assays were performed at different temperature (22 vs. 15°C), the measures of ATP\(_{\text{iso}}\) were adjusted to 22°C based on the Q\(_{10}\) of ATP\(_{\text{iso}}\) so that comparisons with V\(_{\text{max}}\) ATPase could be made. The procedures for this correction have been previously described in detail (19). Briefly, in a subset of permeabilized DIAm fibers, the temperature dependence (temperature coefficient Q\(_{10}\)) of ATP\(_{\text{iso}}\) was determined by obtaining measurements at 15, 20, and 25°C for 10 single fibers. The Q\(_{10}\) was ~1.3, regardless of fiber type.

Measurement of single-fiber mechanical properties. A computer program described by Fabiato and Fabiato (7), with stability constants listed by Godt and Lindley (15), was used to prepare a high Ca\(^{2+}\) (pCa 4.0) and a low Ca\(^{2+}\) (pCa 9.0) relaxing solution. With an ionic strength of 150 mM at 15°C, both high and low Ca\(^{2+}\) solutions contained the following: 1.0 mM free Mg\(^{2+}\), 10 mM EGTA, 15 mM CrP, 50 mMimidazole, 2 mM DTT, 5 mM NaATP, and creatine phosphokinase at 1 mg/ml.

A 5% gluteraldehyde solution was used to chemically fix the ends of the fiber to maintain the noncontractile attachment of the fiber to the force transducer and the servo-controlled motor. To further reduce compliance and allow for fiber mounting, aluminum foil T-clips were attached to the fixed end of the fiber. On an Olympus IMT-2 inverted microscope, fibers were mounted on two stainless steel hooks between a force transducer (Aksjeselskapet, AE-801) with a resonant frequency of 5 kHz and a servo-motor (General Scanning, G120DT), with a step time of 800 μs in a flow-through acrylic chamber (volume, 120 μl). Fiber length (10× Olympus Plan 10, 0.30 numerical aperture), width (XY plane), and depth (Z plane) (40× Olympus LWD CD Plan 40, 0.55 numerical aperture) were measured using a reticule in the eye piece of the inverted microscope. As stated in a previous study (13), a correction factor (0.867) was used to account for the distortion of the Z-axis due to the optics. Using first-order laser diffraction (He-Ne laser, UDT Sensors, LSC 30D), the fiber sarcomere length was set to 2.5 μm, where the muscle fiber was at the optimal fiber length (L\(_o\)). Bremer cycling (4), as modified by Sweeney (51), was used to maintain the sarcomere length during the experiment. LabView (National Instruments) based software and data acquisition boards were used to record signals. The experimental setup was used to measure the following: 1) the α\(_{κf} 2) V_o, and 3) the rate constant for force redevelopment (k\(_{FR}\)).

The method employed to estimate the α\(_{κf}\) has been previously described in detail (3, 4, 13, 14, 34–37, 39, 52). Briefly, permeabilized fibers were exposed to a pCa 9.0 solution to determine baseline force. Fibers were then maximally activated using pCa 4.0 solution. During maximum activation, muscle fiber stiffness was determined by imposing small-amplitude (≤0.2% L\(_o\)) high-frequency (2 kHz) sinusoidal length perturbations and measuring the resulting changes in force. The measurement of muscle stiffness assumes that these small-amplitude, high-frequency length perturbations do not disrupt cross-bridge binding and exceed normal cross-bridge cycling rate. In this case, each strongly bound cross bridge contributes incrementally to the measured stiffness. In the present study, fiber stiffness was measured during maximal Ca\(^{2+}\) activation (pCa 4.0) in the presence or absence (rigor) of ATP. It was assumed that stiffness during rigor conditions represents the maximum number of strongly bound cross bridges. Accordingly, the ratio of fiber stiffness in the presence and absence of ATP provided an estimate of α\(_{κf}\) during maximal Ca\(^{2+}\) activation.

V\(_o\). While fibers were maximally activated, V\(_o\) was determined using the slack test (6, 28) during which fiber length was rapidly shortened using length steps ranging from 5 to 11% of L\(_o\). After each length step, the time required for the fiber to redevelop force (slack time) provides a measure of V\(_o\). A linear regression of the different length steps versus slack times was used to determine V\(_o\) (expressed as muscle length/s).

k\(_{FR}\). Cross-bridge cycling rate was estimated for each fiber by measuring the rate constant for force redevelopment (k\(_{FR}\)) using a method previously described in detail (5, 13, 14, 34, 35, 37, 39, 41, 52). Briefly, during maximum Ca\(^{2+}\) activation, fiber length was rapidly shortened (by ~20% of L\(_o\)) and then restretched to L\(_o\). This resulted in detachment of all cross bridges, and force was reduced to baseline (zero). Thereafter, as cross bridges reattached, force redeveloped, and the rate of rise of force reflected cross-bridge cycling rate within the fiber. A computer algorithm for least squares fit of a first-order exponential was used to determine the k\(_{FR}\). Since k\(_{FR}\) depends on both f\(_{app}\) and g\(_{app}\) (Eq. 3), this relationship can be used to estimate these parameters.
Equations 7 and 8 describe how an approximation of \( f_{\text{app}} \) is obtained from the values of \( \alpha_s \) and \( k_{\text{TR}} \):\[ k_{\text{TR}} = f_{\text{app}} + g_{\text{app}} \] (6)

\[ \alpha_s = \frac{f_{\text{app}}}{f_{\text{app}} + g_{\text{app}}} \] (7)

\[ f_{\text{app}} = \alpha_s k_{\text{TR}} \] (8)

By subtracting the value of \( f_{\text{app}} \) from \( k_{\text{TR}} \) as in Eq. 9, an approximation of \( g_{\text{app}} \) can be obtained:

\[ g_{\text{app}} = k_{\text{TR}} - f_{\text{app}} \] (9)

By using the equations listed above, the cross-bridge cycling kinetics for single fibers expressing different MHC isoforms in the rat DI\( \text{Am} \) can be examined.

**Determination of MHC isoform expression in single fibers by gel electrophoresis.** MHC isoform expression in single fibers was determined post hoc by SDS-PAGE (13, 14). The single fibers were placed in 25 \( \mu \)l of SDS sample buffer containing 62.5 mM Tris-HCl, 2\% (wt/vol) SDS, 10\% (vol/vol) glycerol, 5\% 2-mercaptoethanol, and 0.001\% (wt/vol) bromophenol blue at a pH of 6.8. The samples were denatured by boiling for 2 min. A modified procedure by Sugiura and Murakami (50) was used to prepare the gradient gels. The stacking gel contained a 3.5\% acrylamide concentration (pH 6.8), and the separating gel contained 5–8\% acrylamide (pH 8.8) with 25\% glycerol (8 \( \times \) 10 cm, 0.75 mm thick; Hoefer SE 250). Ten-microliter samples were loaded per lane. The gels were stained with silver, according to the procedure described by Oakley et al. (30). Control samples of DI\( \text{Am} \) bundles in a 1:200 dilution of SDS sample buffer were run with the single fibers for comparison of migration patterns of the MHC isoforms. Western blot analysis was also performed to confirm this identification of MHC isoforms by migration patterns (13, 14, 53).

**Measurement of MHC concentration in single fibers.** The methods used to measure MHC concentration in single DI\( \text{Am} \) fibers have been previously described in detail (13, 14, 53). Briefly, known concentrations of purified rabbit MHC (Sigma M-3889) were separated on the same gels as those used for detecting MHC in single fibers. The gels were silver stained, and a high-resolution scanner (Microtek ScanMaker 5, 600 dpi) was used for densitometric analysis. The brightness area product of each rabbit MHC sample was determined from the linear relationship between brightness and average brightness of each band following subtraction of local background. Based on the linear relationship between brightness area product and MHC concentration in these standard samples, the MHC concentration in single DI\( \text{Am} \) fibers was determined.

**Statistical analysis.** The effects of unilateral DI\( \text{Am} \) DNV on \( V_{\text{max}} \), ATPase, ATP\(_{\text{max}}\), \( \alpha_s \), \( V_o \), and \( k_{\text{TR}} \) were analyzed by using a two-way ANOVA. When appropriate, post hoc analysis (Student’s \( t \)-test) was also used. Differences were considered as significant at \( P < 0.05 \). All data are presented as means \( \pm \) SE.

**RESULTS**

**MHC isoform expression in DI\( \text{Am} \) fibers.** On the basis of differences in electrophoretic migration, four MHC isoforms were identified: MHC\(_{\text{slow}}\), MHC\(_{2A}\), MHC\(_{2X}\), and MHC\(_{2B}\). In single fibers from CTL DI\( \text{Am} \), MHC\(_{\text{slow}}\), MHC\(_{2A}\), and MHC\(_{2X}\) were all observed to be singularly expressed, while expression of the MHC\(_{2B}\) isoform was only observed together with MHC\(_{2X}\) (Fig. 1). The expression of four different MHC isoforms was also detected by immunohistochemistry, although the coexpression of MHC\(_{2X}\) and MHC\(_{2B}\) isoforms could not be unambiguously identified.

**Effects of DNV on relative MHC isoform expression.** After 2 wk of DNV, changes in the expression patterns of MHC isoforms were observed. Whereas some DI\( \text{Am} \) fibers continued to singularly express MHC\(_{\text{slow}}\), MHC\(_{2A}\), and MHC\(_{2X}\) isoforms, an increased incidence of coexpression of MHC\(_{\text{slow}}\) and MHC\(_{2A}\) and MHC\(_{2A}\) and MHC\(_{2X}\) was observed (Table 1). Following DNV, expression of MHC\(_{2B}\) in DI\( \text{Am} \) fibers was always observed, together with MHC\(_{2X}\) (coexpression, top). Following DNV, expression of MHC\(_{2B}\) in DI\( \text{Am} \) fibers was not detected, and the incidence of coexpression of MHC\(_{\text{slow}}\) with MHC\(_{2A}\) and MHC\(_{2A}\) with MHC\(_{2X}\) isoforms increased (not shown). The concentration of MHC extracted from single DI\( \text{Am} \) fibers was determined by comparison to known concentrations of commercially available MHC run in the same gel (bottom). The dimensions of the single fiber (cross-sectional area and length – number of sarcomeres in series) were measured, and MHC content per half-sarcomere was calculated based on these measurements.

![Fig. 1. Electrophoretic determination of myosin heavy chain (MHC) isoform expression in single rat diaphragm muscle (DI\( \text{Am} \)) fibers. Most DI\( \text{Am} \) fibers in control (CTL) displayed singular expression of MHC\(_{\text{slow}}\), MHC\(_{2A}\), and MHC\(_{2X}\) isoforms. Expression of MHC\(_{2B}\) in DI\( \text{Am} \) fibers was always observed, together with MHC\(_{2X}\) (coexpression, top). Following DNV, expression of MHC\(_{2B}\) in DI\( \text{Am} \) fibers was not detected, and the incidence of coexpression of MHC\(_{\text{slow}}\) with MHC\(_{2A}\) and MHC\(_{2A}\) with MHC\(_{2X}\) isoforms increased (not shown). The concentration of MHC extracted from single DI\( \text{Am} \) fibers was determined by comparison to known concentrations of commercially available MHC run in the same gel (bottom). The dimensions of the single fiber (cross-sectional area and length – number of sarcomeres in series) were measured, and MHC content per half-sarcomere was calculated based on these measurements.](image-url)
content per half-sarcomere ($n$ in Eq. 1) was reduced in DIAm fibers expressing MHC$_{2X}$ ($P < 0.05$, Table 2).

**Effect of DNV on $V_{\text{max}}$ ATPase.** In CTL DIAm, $V_{\text{max}}$ ATPase was significantly higher in fibers expressing MHC$_{2X}$ and MHC$_{2A}$ compared with those expressing MHC$_{\text{slow}}$ and MHC$_{2A}$ (Fig. 3; $P < 0.05$). After 2 wk of DNV, $V_{\text{max}}$ ATPase was significantly reduced (Fig. 3; $P < 0.05$) in all DIAm fibers, but to a greater extent in fibers displaying immunoreactivity for the anti-MHC$_{2B}$ antibody or the absence of immunoreactivity for the anti-MHC$_{\text{all-2X}}$ antibody ($P < 0.05$). As a result, no significant differences were found across the fibers expressing different MHC isoforms after DNV (Fig. 3).

**Effect of DNV on ATP$_{\text{iso}}$.** In CTL DIAm, ATP$_{\text{iso}}$ was significantly slower in fibers expressing MHC$_{\text{slow}}$ and MHC$_{2A}$ compared with those expressing MHC$_{2X}$ alone or together with MHC$_{2B}$ (Fig. 4A; $P < 0.05$). After 2 wk of DNV, ATP$_{\text{iso}}$ was significantly reduced in fibers expressing MHC$_{2X}$ (Fig. 4A; $P < 0.05$). As a result, no significant differences in ATP$_{\text{iso}}$ were found across DIAm fibers after DNV.

ATP$_{\text{iso}}$ per MHC content. ATP$_{\text{iso}}$ was normalized to MHC content to evaluate the effect of cross-bridge number on ATP consumption rate. In CTL DIAm, ATP$_{\text{iso}}$ per MHC content was significantly lower in fibers expressing MHC$_{\text{slow}}$ compared with fibers expressing MHC$_{2A}$, MHC$_{2X}$ alone, or MHC$_{2X}$ together with MHC$_{2B}$ (Fig. 4B; $P < 0.05$). In particular, ATP$_{\text{iso}}$ per MHC content was higher in DIAm fibers coexpressing MHC$_{2X}$ and MHC$_{2B}$ isoforms. After 2-wk DNV, ATP$_{\text{iso}}$ per MHC content was significantly reduced in DIAm fibers expressing MHC$_{2X}$ (Fig. 4B; $P < 0.05$).

**Reserve capacity.** In CTL DIAm, the difference between $V_{\text{max}}$ ATPase and ATP$_{\text{iso}}$, reflecting the reserve capacity for ATP consumption, was higher for fibers expressing MHC$_{\text{slow}}$ and MHC$_{2A}$ compared with fibers expressing MHC$_{2X}$ alone or together with MHC$_{2B}$ (Fig. 5; $P < 0.05$). After 2 wk of DNV, the reserve capacity for ATP consumption was significantly reduced across all DIAm fibers, but to a greater extent in fibers expressing MHC$_{2X}$ (Fig. 5; $P < 0.05$).

$V_o$. In CTL DIAm, $V_o$ varied across fibers expressing different MHC isoforms (Fig. 6A; $P < 0.05$). Fibers coexpressing MHC$_{2X}$ and MHC$_{2B}$ and singularly expressing MHC$_{2X}$ were

### Table 1. Effect of DNV on MHC isoform expression in single diaphragm muscle fibers

<table>
<thead>
<tr>
<th>MHC Isoform</th>
<th>CTL, no. (%)</th>
<th>DNV, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>30 (21%)</td>
<td>32 (29%)</td>
</tr>
<tr>
<td>2A</td>
<td>27 (19%)</td>
<td>20 (18%)</td>
</tr>
<tr>
<td>Slow/2A</td>
<td>0</td>
<td>21 (19%)</td>
</tr>
<tr>
<td>2A/2X</td>
<td>22 (20%)</td>
<td></td>
</tr>
<tr>
<td>2X</td>
<td>45 (32%)</td>
<td>13 (12%)</td>
</tr>
<tr>
<td>2X/2B</td>
<td>38 (27%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>111</td>
</tr>
</tbody>
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MHC, myosin heavy chain; CTL, control; DNV, denervation.

### Table 2. Effect of DNV on MHC content per half-sarcomere in single diaphragm muscle fibers

<table>
<thead>
<tr>
<th>MHC Isoform</th>
<th>CTL</th>
<th>DNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>1.84±0.10</td>
<td>1.59±0.26</td>
</tr>
<tr>
<td>2A</td>
<td>1.86±0.33</td>
<td>1.89±0.27</td>
</tr>
<tr>
<td>Slow/2A</td>
<td>2.04±0.27</td>
<td>2.02±0.34</td>
</tr>
<tr>
<td>2X</td>
<td>4.10±0.53*</td>
<td>2.68±0.13*</td>
</tr>
<tr>
<td>2X/2B</td>
<td>5.79±0.68*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in μg × 10⁻⁴. *Significant difference from MHC$_{\text{slow}}$ ($P < 0.05$). †Significant difference ($P < 0.05$) between CTL and DNV.

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Fig. 2. Cross-sectional areas of DIAm fibers expressing MHC$_{2X}$ decreased following 2 wk of denervation (DNV). In CTL DIAm, fibers expressing MHC$_{\text{slow}}$ and MHC$_{2A}$ were smaller than those expressing MHC$_{2X}$ alone or together with MHC$_{2B}$. Following 2 wk of DNV, cross-sectional areas of fibers expressing MHC$_{\text{slow}}$ and MHC$_{2X}$ increased slightly, whereas fibers expressing MHC$_{2X}$ atrophied. Expression of MHC$_{2A}$ was not detected in single fibers after DNV. However, the incidence of coexpression of MHC$_{\text{slow}}$ and MHC$_{2A}$, and MHC$_{2A}$ and MHC$_{2X}$ isoforms increased in the DNV DIAm. *Significant difference ($P < 0.05$) compared with fibers expressing MHC$_{\text{slow}}$. #Significant difference ($P < 0.05$) between CTL and DNV.

Fig. 3. The maximum velocity ($V_{\text{max}}$) of the actomyosin ATPase reaction in single DIAm fibers expressing different MHC isoforms was measured using a quantitative histochemical technique. In CTL DIAm, $V_{\text{max}}$ was significantly higher in fibers expressing MHC$_{2X}$ alone or together with MHC$_{2B}$ compared with fibers expressing MHC$_{\text{slow}}$ and MHC$_{2X}$. After 2 wk of DNV, there was significant decrease in $V_{\text{max}}$ across all fibers, but this effect was most pronounced in fibers expressing MHC$_{2X}$. *Significant difference ($P < 0.05$) compared with fibers expressing MHC$_{\text{slow}}$. #Significant difference ($P < 0.05$) between CTL and DNV.
Effect was more pronounced for fibers expressing MHC2X (Fig. 6B; P < 0.05). MHC2X isoform in these fibers (typically increased after DNV. ATPiso (measured at 15°C) varied with MHC isoform of fibers expressing MHC2X. *Significant difference (P < 0.05) from fibers expressing MHCslow. #Significant difference (P < 0.05) from CTL within a fiber type.

MHCslow (Fig. 7; P < 0.05). After 2 wk of DNV, both fapp and gapp slowed across all DIam fibers (Fig. 7; P < 0.05), but this slowing was more pronounced for fibers expressing MHC2X (Fig. 7; P < 0.05).

**DISCUSSION**

The results of the present study indicate that unilateral DNV results in a selective reduction of ATPiso in DIam fibers expressing MHC2X, whereas the ATPiso of DIam fibers expressing MHCslow and MHC2A were relatively unaffected. Two weeks of DNV caused atrophy of DIam fibers expressing MHC2X and a decrease in MHC content per half-sarcomere (n in Eq. 1) in these fibers. Thus the effect of DNV on ATPiso in fibers expressing MHC2X can be explained, at least in part, by a decrease in the number of cross bridges contributing to ATP consumption (see Eq. 1). However, when ATPiso was normalized for the DNV-induced decrease in MHC content, the effect on ATP consumption in fibers expressing MHC2X persisted. After 2 wk of DNV, there was also a slowing of cross-bridge cycling rate (reflected by slower Vapp and kTR) in DIam fibers that was most pronounced in fibers expressing MHC2X. A slowing of cross-bridge cycling rate after DNV may have also contributed to the reduction in ATPiso in DIam fibers expressing MHC2X (decreased gapp in Eq. 1).

**Effect of DNV on DIam MHC isoform expression and cross-sectional area.** In agreement with previous studies, we found that DNV affected the expression of different MHC isoforms. Most striking was the markedly reduced expression found to have ~50% faster Vapp compared with fibers expressing MHCslow and MHC2A (Fig. 6A; P < 0.05). After 2 wk of DNV, Vapp slowed across all DIam fibers (Fig. 6A; P < 0.05), but this effect was more pronounced for fibers expressing MHC2X (Fig. 6A; P < 0.05).

kTR. In CTL DIam, kTR varied across fibers expressing different MHC isoforms, with a rank order of MHC2X and MHC2B coexpress > MHC2X > MHC2A > MHCslow (Fig. 6B; P < 0.05). After 2 wk of DNV, kTR was slower across all DIam fibers (Fig. 6B; P < 0.05), but this effect was more pronounced for fibers expressing MHC2X (Fig. 6B; P < 0.05). In DIam fibers, the kTR for coexpressing MHCslow and MHC2A was intermediate to that for fibers singularly expressing these isoforms. In DIam fibers coexpressing MHC2A and MHC2X, kTR was closer to that observed in fibers singularly expressing MHC2X. This may reflect the greater relative abundance of the MHC2X isoform in these fibers (typically >70% MHC2X compared with <30% MHC2A).

fapp and gapp. In CTL DIam, fapp and gapp varied across fibers expressing different MHC isoforms, with a rank order of MHC2X and MHC2B coexpress > MHC2X > MHC2A > MHC2B coexpress.

Table 3. Effect of DNV on αf, in single diaphragm muscle fibers

<table>
<thead>
<tr>
<th>MHC Isoform</th>
<th>CTL</th>
<th>DNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>0.77 ± 0.10</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td>2A</td>
<td>0.81 ± 0.05</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>Slow/2A</td>
<td>0.81 ± 0.05</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>2A/2X</td>
<td>0.74 ± 0.07</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td>2X</td>
<td>0.73 ± 0.05</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td>2X/2B</td>
<td>0.79 ± 0.02</td>
<td>0.78 ± 0.06</td>
</tr>
</tbody>
</table>

αf. Fraction of available cross bridges that are in a strongly bound state.
of the MHC2B isoform and the increased incidence of coexpression of MHC isoforms. In the rate DIAm, the MHC2B isoform is typically coexpressed in varying proportion with the MHC2X isoform (10–12). Clearly, MHC isoform coexpression affected ATPiso, since values in fibers coexpressing MHC isoforms were generally intermediate to those observed in fibers singularly expressing MHC isoforms. However, MHC isoform transitions and coexpression alone cannot explain the results of the present study, since both the ATPiso of fibers singularly expressing the MHC2X isoform were reduced following DNV.

Previous studies in several species have reported a transient hypertrophy of DI Am fibers following unilateral DNV (16, 20, 38, 47, 48), with fiber-type-dependent differences in morphological adaptations appearing after 1–2 wk (56–59). Following DNV, DI Am fibers expressing MHCslow and MHC2A remain slightly hypertrophied, whereas fibers expressing MHC2X display selective atrophy. Similar selective fiber-type-dependent atrophy of DI Am fibers has been noted in a number of other physiological and pathological conditions, such as corticosteroid treatment (25, 40, 54), hypothyroidism (11, 16, 44), heart failure (21), and under-nutrition (24, 26, 27, 40). A decrease in MHC content per half-sarcomere (reduced number of cross bridges, n in Eq. 1) should result in a reduction in ATPiso, which was observed in DI Am fibers expressing MHC2X following DNV. Similarly, we found that a decrease in MHC content per half-sarcomere induced by DNV (10) or hypothyroidism (11) is associated with reduced maximum isometric force generated by DI Am fibers expressing MHC2X.

Effect of DNV on ATPiso and cross-bridge cycling rate. MHC is the site of ATP hydrolysis during cross-bridge cycling, and ATP consumption rate is a major determinant of muscle fiber mechanical performance, as evidenced by the close relationship between \( V_{\text{max}} \) ATPase (measured biochemically) and fiber-type composition and contractile properties of various skeletal muscles (1). We and others have used an NADH-linked fluorometric technique to measure ATP consumption rates in single permeabilized muscle fibers during maximum isometric activation (i.e., ATPiso) (3, 19, 39, 42). In general, in different muscles, it has been shown that fibers expressing MHC2X have slower ATPiso compared with fibers expressing MHCslow and MHC2A, MHC2X, and MHC2B isoforms. However, ATPiso is submaximal and does not establish maximum capacity for ATP hydrolysis (19, 39, 42). Furthermore, ATP consumption increases with power output and work performance (Fenn effect) (8, 9, 39). Thus, as fibers shorten and reach maximum power, ATP consumption rate increases (39). We have previously established in the rat DI Am that the maximum rate of ATP consumption is achieved at a shortening velocity corresponding to peak power output of DI Am fibers. Accordingly, \( V_{\text{max}} \) ATPase establishes the upper limit for ATP consumption for each fiber type. The difference in ATP consumption rates from

![Fig. 6](https://example.com/fig6.png)

A: effect of DNV on maximum unloaded shortening velocity (\( V_o \)). \( V_o \) was significantly higher in fibers expressing MHC2X alone or together with MHC2B isoforms compared with fibers expressing MHCslow and MHC2A in CTL DI Am. DNV resulted in significant decrease in \( V_o \) across all fibers, but this effect was more pronounced in fibers expressing MHC2X. B: effect of DNV on cross-bridge cycling rate (\( k_{TB} \)). Significant differences in \( k_{TB} \) were found across all fibers expressing different MHC isoforms in both CTL and DNV DI Am. DNV resulted in significant decrease in \( k_{TB} \) across all fibers. *Significant difference (\( P < 0.05 \)) from fibers expressing MHCslow. #Significant difference (\( P < 0.05 \)) from CTL within a fiber type.

![Fig. 7](https://example.com/fig7.png)

A: effect of DNV on apparent rate constants for cross-bridge attachment (\( f_{app} \)) and detachment (\( g_{app} \)). Significant differences in both \( f_{app} \) and \( g_{app} \) were found across all fibers expressing different MHC isoforms in CTL DI Am. DNV resulted in significant decrease in both \( f_{app} \) and \( g_{app} \) across all fibers. *Significant difference (\( P < 0.05 \)) from fibers expressing MHCslow. #Significant difference (\( P < 0.05 \)) from CTL within a fiber type.
ATP$_{iso}$ to $V_{\text{max}}$ ATPase provides a measure of the reserve capacity for ATP consumption in muscle fibers. The difference in ATP consumption rates from ATP$_{iso}$ to $V_{\text{max}}$, ATPase provides a measure of the reserve capacity for ATP consumption in muscle fibers. In this study, we found that the reserve capacity for ATP consumption was $-65$, $-55$, and $-50\%$ for fibers expressing MHC$_{\text{slow}}$, MHC$_{2A}$, and MHC$_{2X}$ alone or together with MHC$_{2B}$, respectively. DNV significantly reduced the reserve capacity for ATP consumption across all DIAm fibers, but to a greater extent in fibers expressing MHC$_{2X}$.

According to Eq. 1, a decrease in ATP$_{iso}$ could result from a decrease in the number of cross bridges per half-sarcomere ($n$, MHC content per half-sarcomere), a decrease in $g_{\text{app}}$ (as reflected by a slowing of cross-bridge cycling, $k_{TR}$ or $V_o$), or a decrease in the $\alpha$$_f$. Following 2 wk of DNV, there was a reduction in MHC content per half-sarcomere ($n$) in DIAm fibers expressing MHC$_{2X}$. When ATP$_{iso}$ was normalized for this DNV-induced reduction in MHC content, ATP consumption per MHC molecule was still decreased in fibers expressing MHC$_{2X}$. Following 2 wk of DNV, cross-bridge cycling rate was slower in fibers expressing MHC$_{2X}$ (as reflected by slower $k_{TR}$ and $V_o$); thus a decrease in $g_{\text{app}}$ could also account for the reduction in ATP$_{iso}$ in DIAm fibers expressing MHC$_{2X}$. Finally, the $\alpha$$_f$ was unaffected by DNV. Together, these results support the conclusion that DNV reduces ATP$_{iso}$ in DIAm fibers expressing MHC$_{2X}$ by reducing MHC content and by slowing cross-bridge cycling rate (i.e., $g_{\text{app}}$).

The underlying mechanism(s) by which DNV affects selective changes in MHC content and cross-bridge cycling in DIAm fibers expressing MHC$_{2X}$ is unclear. One possibility is that the removal of neurotransphin influence following DNV leads to a selective atrophy of DIAm fibers expressing MHC$_{2X}$, and that the reduction in MHC content per half-sarcomere leads to altered myofilament lattice spacing that, in turn, affects cross-bridge cycling rate and ATP consumption.

Clinical relevance. Although infrequent, DIAm DNV may occur during cardiac or thoracic surgery. However, the DNV model also relates to other physiological and pathological conditions that are associated with decreased muscle mass, reduced MHC content, and/or muscle weakness, such as sarcopenia, corticosteroid treatment, heart failure, chemotherapy, under-nutrition, and hypothyroidism. The significance of the results of the present study should not be narrowly limited to DNV. What is important is that DNV induces a decrease in MHC concentration in muscle fibers. Since MHC is the site of ATP hydrolysis during muscle activation and cross-bridge cycling, a decrease in MHC concentration should result in a decrease in ATP consumption during muscle activation (i.e., a decrease in ATP$_{iso}$). Indeed, a decrease in ATP$_{iso}$ was observed, but to a greater extent than that predicted by the decrease in MHC concentration alone: ATP$_{iso}$ normalized for MHC concentration was still reduced in type IIX fibers. Thus factors in addition to the decrease in MHC concentration must also play a role. Indeed, DNV was found to induce a slowing of $V_o$ and $k_{TR}$, indicating a slowing of cross-bridge cycling rate that was more pronounced in type IIX fibers.

With corticosteroid treatment (25, 54, 55), hypothryroidism (11, 16, 44), heart failure (21), and under-nutrition (24, 26, 27, 40), there is also DIAm weakness and selective atrophy of fiber expressing MHC$_{2X}$ alone or together with MHC$_{2B}$. Thus selective effects on fibers expressing MHC$_{2X}$ and/or MHC$_{2B}$ may extend to other models where atrophy and muscle weakness are found. Whether or not there are changes in MHC concentration that affect ATP consumption rate has not been explored in these conditions.

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GRANTS

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REFERENCES


