Effect of denervation on ATP consumption rate of diaphragm muscle fibers

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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 MHC2X isoform but not in fibers expressing MHCslow 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 NAHD-linked fluorometry. The rate of ATP consumption during maximum unloaded shortening velocity (Vmax) and cross-bridge cycling rate [estimated from the rate constant for force redevelopment (kTR) 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, Vmax and kTR were slowed in fibers expressing MHC2X, consistent with the effect on ATPiso. The difference between Vmax 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 Vmax 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.

myosin heavy chain; muscle energetics; muscle plasticity

MYOSIN HEAVY CHAIN (MHC) IS THE SITE OF ATP HYDROLYSIS DURING CROSS-BRIDGE CYCLING, AND ATP CONSUMPTION RATE DURING CROSS-BRIDGE CYCLING IS A MAJOR DETERMINANT OF THE MECHANICAL PERFORMANCE OF SKELETAL MUSCLE FIBERS. CROSS BRIDGES CYCLE BETWEEN A FORCE-GENERATING STATE, DURING WHICH CROSS BRIDGES ARE STRONGLY ATTACHED TO ACTIN, AND A NON-FORCE-GENERATING STATE, DURING WHICH CROSS BRIDGES ARE DETACHED FROM ACTIN (22, 23). THE TRANSITIONS BETWEEN THESE TWO FUNCTIONAL STATES ARE DESCRIBED BY TWO APPARENT RATE CONSTANTS: ONE FOR CROSS-BRIDGE ATTACHMENT (gapp) AND THE OTHER FOR CROSS-BRIDGE DETACHMENT (gapp). ATP CONSUMPTION RATE DURING CROSS-BRIDGE CYCLING IS DESCRIBED BY THE FOLLOWING EQUATION:

\[ \text{ATP consumption rate} = b \cdot n \cdot g_{\text{app}} \cdot \alpha_{f} \] (1)

where \( b \) is the number of half-sarcomeres within a muscle fiber, \( n \) is the number of available cross bridges per half-sarcomere, and \( \alpha_{f} \) is the fraction of available cross bridges that are in a strongly bound state.

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 MHC2X 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 (\( b \cdot n \) from Eq. 1), but, even when normalized for MHC content, ATPiso of fibers expressing MHC2X is lower than that of fibers expressing fast MHC isoforms (18). During maximum calcium activation, the \( \alpha_{f} \) is comparable across all fiber types, but there are differences in maximum shortening velocity (reflecting cross-bridge cycling rate and \( g_{\text{app}} \)) that contribute to the fiber-type differences in ATPiso (18).

The maximum velocity of the actomyosin ATPase reaction (Vmax 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 Vmax 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 Vmax ATPase, which is expected, since ATP consumption rate increases during shortening and work performance in muscle (Fenn effect) (8, 9). The difference between Vmax 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 (V0) 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 (gapp) of individual fibers. In addition, DNV leads to a decrease in Vmax 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

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cross-bridge cycling rate and ATP$_{iso}$ that is more pronounced in fibers expressing MHC$_{2X}$.

**METHODS**

Adult male Sprague-Dawley rats (initial body weight $\sim$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 3rd day 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 DiaAm DNV.** The procedures for unilateral DNV of the rat DiaAm 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 DiaAm 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, polymyxin, 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 DiaAm 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 DiaAm fiber size, on MHC content in single DiaAm fibers, on DiaAm force, and velocity of shortening (10, 12, 29, 58, 59).

**Measurement of V$_{max}$ ATPase in single fibers.** The quantitative histochemical procedure for measuring the V$_{max}$ ATPase in type-identified muscle fibers has been previously described in detail (2, 43, 46, 58). Briefly, segments from the right midcostal DiaAm 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 $-20^\circ$C (model 2800E Frigocut, Reichert-Jung).

To determine V$_{max}$ ATPase in single muscle fibers, the reaction product of the actomyosin ATPase reaction (amount of P$_i$ 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 sulfide precipitate reacted with a lead ammonium citrate/acetate complex to form a lead chromate cathod. 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 $\leq$ 1.0, measurement errors were $\leq$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$_{max}$ 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-MHC$_{slow}$ (Novocastra, IgG; 1:100), anti-MHC$_{2X}$ (Blau A4.74, IgG; 1:1), anti-MHC$_{2B}$ (BFF3, IgG; 1:200), anti-MHC$_{1A}$ (B5.1, IgM, purified from mouse hybridoma, German Collection of Microorganisms and Cell Culture; 1:1) and anti-MHC$_{2B}$ (Novocastra; 1:10). To determine MHC$_{2X}$ expression, only a single antibody was used, i.e., anti-MHC$_{2B}$ (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 $\sim$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 DiaAm 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$_2$PO$_4$; 1 mM MgATP, 7 mM EGTA, propionate as the major anion, 10 mM free 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 $-20^\circ$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 ATP

ATP 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:

\[
\text{ATPase} \\
\text{ATP} \rightarrow \text{ADP} + P_i \quad (3)
\]

\[
\text{PK} \\
\text{ADP} + \text{PEP} \rightarrow \text{pyruvate} + \text{ATP} \quad (4)
\]

\[
\text{Pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}^+ \quad \text{(fluorescent)} \quad (5)
\]

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 \(\mu\)M (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 \(\mu\)m, 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 \(1 - \frac{\text{ratio of ATP}_{\text{iso}}}{V_{\text{max}} \text{ ATPase}}\), 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 mM imidazole, 2 mM DTT, 5 mM NaATP, and creatine phosphokinase at 1 mg/ml.

A 5% glutaraldehyde solution was used to chemically fix the ends of the fiber to maintain the noncompliant 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 \(\mu\)s in a flow-through acrylic chamber (volume, 120 \(\mu\)l). Fiber length (10× Olympus Plan 10, 0.30 numerical aperture), width (XY plane), and depth (AZ plane) were also estimated. Based on these measurements, fiber cross-sectional area was used to calculate the rate constant for force redevelopment (\(k_{\text{TR}}\)).

\[\alpha_f\] the method employed to estimate the \(\alpha_f\) 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% \(L_0\), 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 \(\alpha_f\) 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 \(L_0\). 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 vs. slack times was used to determine \(V_o\) (expressed as muscle length/s).

\(k_{\text{TR}}\) Cross-bridge cycling rate was estimated for each fiber by measuring the rate constant for force redevelopment (\(k_{\text{TR}}\)) 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 \(L_0\)) and then restretched to \(L_0\). 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 \(k_{\text{TR}}\). Since \(k_{\text{TR}}\) depends on both \(f_{\text{app}}\) and \(g_{\text{app}}\) (Eq. 3), this relationship can be used to estimate these parameters.

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MHC2x (Fig. 1). The expression of four different MHC isoforms were identified: MHCslow, MHC2A, MHC2X, and MHC2B. In DIAm, MHCslow, MHC2A, and MHC2X were all observed to be singularly expressed, while expression of the MHC2B isoform was only observed together with MHC2X (coexpression; top). Following DNV, expression of MHC2B in DIAm fibers was always observed, together with MHC2X (coexpression; top). Following DNV, expression of MHC2B in DIAm fibers was always observed, together with MHC2X (coexpression; top).

Measurement of MHC concentration in single fibers. The methods used to measure MHC concentration in single DIAm 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 stained with silver, according to the procedure described by Oakley et al. (30). Control samples of DIAm 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 periodically performed to confirm this identification of MHC isoforms by migration patterns (13, 14, 53).

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 μl 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 Sugiyama 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 × 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 DIAm 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 periodically performed to confirm this identification of MHC isoforms by migration patterns (13, 14, 53).

EQN 7 and 8 describe how an approximation of \( f_{app} \) is obtained from the values of \( \alpha_s \) and \( k_{TR} \) (4, 41):

\[
\alpha_s = \frac{f_{app} \cdot g_{app}}{f_{app} + g_{app}}
\]

\[
f_{app} = \alpha_s k_{TR}
\]

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

\[
g_{app} = k_{TR} - f_{app}
\]

By using the equations listed above, the cross-bridge cycling kinetics for single fibers expressing different MHC isoforms in the rat DIAm can be examined.

\[\text{RESULTS}\]

MHC isoform expression in DIAm fibers. On the basis of differences in electrophoretic migration, four MHC isoforms were identified: MHCslow, MHC2A, MHC2X, and MHC2B. In single fibers from CTL DIAm, MHCslow, MHC2A, and MHC2X were all observed to be singularly expressed, while expression of the MHC2B isoform was only observed together with MHC2X (Fig. 1). The expression of four different MHC isoforms was also detected by immunohistochemistry, although the coexpression of MHC2X and MHC2B 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 DIAm fibers continued to singularly express MHCslow, MHC2A, and MHC2X isoforms, an increased incidence of coexpression of MHCslow and MHC2A and MHC2X isoforms was observed (not shown). The concentration of MHC extracted from single DIAm 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.

Effects of DNV on MHC content. In CTL DIAm, the cross-sectional area of fibers expressing MHCslow and MHC2A isoforms was significantly smaller than those expressing MHC2X alone or together with MHC2B (not shown). It should be emphasized that this distribution only represents the proportion of single fibers randomly sampled in the present study.

Following DNV, DIAm fiber-type classification based on the predominant expression of different MHC isoforms was still possible. However, it was not possible to unambiguously distinguish the coexpression of MHC isoforms. It should be noted that some fibers remained immunoreactive for the anti-MHC2B antibody, although the number of fibers was markedly reduced.

Effects of DNV on DIAm fiber cross-sectional area. In CTL DIAm, the cross-sectional area of fibers expressing MHCslow and MHC2A isoforms was significantly smaller than those expressing MHC2X alone or together with MHC2B (Fig. 2; \( P < 0.05 \)). After 2 wk of DNV, the cross-sectional area of fibers expressing MHCslow and MHC2A was slightly greater than CTL, while the cross-sectional areas of fibers expressing MHC2X were significantly smaller (Fig. 2; \( P < 0.05 \)).

Effects of DNV on MHC content. In CTL DIAm, MHC content was significantly lower in fibers expressing MHCslow and MHC2A compared with those expressing MHC2X alone or together with MHC2B (Table 2). After 2 wk of DNV, MHC...
content per half-sarcomere \( (n \text{ in } Eq. \ 1) \) was reduced in DIAm fibers expressing MHC\textsubscript{2X} \( (P < 0.05, \text{ Table 2}) \).

**Effect of DNV on \( V_{\text{max}} \text{ATPase} \).** In CTL DIAm, \( V_{\text{max}} \text{ ATPase} \) was significantly higher in fibers expressing MHC\textsubscript{2X} and MHC\textsubscript{2A} compared with those expressing MHC\textsubscript{slow} and MHC\textsubscript{2A} (Fig. 3; \( P < 0.05 \)). After 2 wk of DNV, \( V_{\text{max}} \text{ ATPase} \) 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\textsubscript{2B} antibody or the absence of immunoreactivity for the anti-MHC\textsubscript{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 \( \text{ATP}_{\text{iso}} \).** In CTL DIAm, \( \text{ATP}_{\text{iso}} \) was significantly slower in fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{2A} compared with those expressing MHC\textsubscript{2X} alone or together with MHC\textsubscript{2B} (Fig. 4A; \( P < 0.05 \)). After 2 wk of DNV, \( \text{ATP}_{\text{iso}} \) was significantly reduced in fibers expressing MHC\textsubscript{2X} (Fig. 4A; \( P < 0.05 \)). As a result, no significant differences in \( \text{ATP}_{\text{iso}} \) were found across DIAm fibers after DNV.

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

**Reserve capacity.** In CTL DIAm, the difference between \( V_{\text{max}} \text{ ATPase} \) and \( \text{ATP}_{\text{iso}} \), reflecting the reserve capacity for ATP consumption, was higher for fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{2A} compared with fibers expressing MHC\textsubscript{2X} alone or together with MHC\textsubscript{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\textsubscript{2X} (Fig. 5; \( P < 0.05 \)).

\( \alpha_{fs} \). During maximum \( \text{Ca}^{2+} \) activation, \( \alpha_{fs} \) values ranged from 0.73 to 0.81 across all fibers (Table 3). No significant differences in \( \alpha_{fs} \) were found across fibers expressing MHC isoforms in either CTL or DNV fibers. Furthermore, no differences in \( \alpha_{fs} \) were seen among fibers from CTL and DNV DIAm.

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

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**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>45 (32%)</td>
<td>13 (12%)</td>
</tr>
<tr>
<td>2X</td>
<td>38 (27%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>2X/2B</td>
<td>0</td>
<td>140</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>111</td>
</tr>
</tbody>
</table>

**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, ( \mu g \times 10^{-4} )</th>
<th>DNV, ( \mu g \times 10^{-4} )</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>4.10 ± 0.33*</td>
<td>2.68 ± 0.13†</td>
</tr>
<tr>
<td>2A/2X</td>
<td>5.79 ± 0.68*</td>
<td></td>
</tr>
<tr>
<td>2X</td>
<td>2.02 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>2X/2B</td>
<td>0.13*†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in \( \mu g \times 10^{-4} \). *Significant difference from MHC\textsubscript{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\textsubscript{2X} decreased following 2 wk of denervation (DNV). In CTL DIAm, fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{2A} were smaller than those expressing MHC\textsubscript{2X} alone or together with MHC\textsubscript{2B}. Following 2 wk of DNV, cross-sectional areas of fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{2X} increased slightly, whereas fibers expressing MHC\textsubscript{2X} atrophied. Expression of MHC\textsubscript{2A} was not detected in single fibers after DNV. However, the incidence of coexpression of MHC\textsubscript{slow} and MHC\textsubscript{2A}, and MHC\textsubscript{2A} and MHC\textsubscript{2X} isoforms increased in the DNV DIAm. *Significant difference \( (P < 0.05) \) compared with fibers expressing MHC\textsubscript{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\textsubscript{2X} alone or together with MHC\textsubscript{2B} compared with fibers expressing MHC\textsubscript{slow} and MHC\textsubscript{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\textsubscript{2X}. *Significant difference \( (P < 0.05) \) compared with fibers expressing MHC\textsubscript{slow}. #Significant difference \( (P < 0.05) \) between CTL and DNV.
Effect was more pronounced for fibers expressing MHC2X (Fig. 5B).

MHC2X isoform in these fibers (typically increased after DNV. ATPiso (measured at 15°C) varied with MHC isoform of fibers expressing MHC slow. *Significant difference (P < 0.05) from fibers expressing MHC slow. #Significant difference (P < 0.05) from CTL within a fiber type.

MHC slow (Fig. 7; P < 0.05). After 2 wk of DNV, both f app and g app 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 ATP iso in DIAm fibers expressing MHC2X, whereas the ATP iso of DIAm fibers expressing MHC slow 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 ATP iso 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 ATP iso 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 V o 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 ATP iso in DIAm fibers expressing MHC2X (decreased g app 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 V o compared with fibers expressing MHC slow and MHC2A (Fig. 6A; P < 0.05). After 2 wk of DNV, V o 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 > MHC slow (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 DNV fibers, the kTR for coexpressing MHC slow and MHC2A was intermediate to that for fibers singularly expressing these isoforms. In DNV 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).

f app and g app. In CTL DIAm, f app and g app varied across fibers expressing different MHC isoforms, with a rank order of MHC2X and MHC2B coexpress > MHC2X > MHC2A >

Table 3. Effect of DNV on α f, 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>

α f, 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 DIAm 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, DIAm fibers expressing MHCslow and MHC2A in CTL DIAm. 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_TR). Significant differences in k_TR were found across all fibers expressing different MHC isoforms in both CTL and DNV DIAm. DNV resulted in significant decrease in k_TR across all fibers. *Significant difference (P < 0.05) from fibers expressing MHCslow. #Significant difference (P < 0.05) from CTL within a fiber type.

which was observed in DIAm 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 DIAm 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_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 MHCslow have slower ATPiso compared with fibers expressing 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 DIAm that the maximum rate of ATP consumption is achieved at a shortening velocity corresponding to peak power output of DIAm fibers. Accordingly, V_max ATPase establishes the upper limit for ATP consumption for each fiber type. The difference in ATP consumption rates from

Fig. 6. 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 DIAm. 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_TR). Significant differences in k_TR were found across all fibers expressing different MHC isoforms in both CTL and DNV DIAm. DNV resulted in significant decrease in k_TR across all fibers. *Significant difference (P < 0.05) from fibers expressing MHCslow. #Significant difference (P < 0.05) from CTL within a fiber type.

DNV resulted in significant decrease in both f_app and g_app across all fibers (3, 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 DIAm that the maximum rate of ATP consumption is achieved at a shortening velocity corresponding to peak power output of DIAm fibers. Accordingly, V_max ATPase establishes the upper limit for ATP consumption for each fiber type. The difference in ATP consumption rates from

Fig. 7. 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 DIAm. 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\textsubscript{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\textsubscript{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\textsubscript{slow}, MHC\textsubscript{2A}, and MHC\textsubscript{2X} alone or together with MHC\textsubscript{2B}, respectively. DNV significantly reduced the reserve capacity for ATP consumption across all DIAm fibers, but to a greater extent in fibers expressing MHC\textsubscript{2X}.

According to Eq. 1, a decrease in ATP\textsubscript{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 $\alpha_{fs}$. Following 2 wk of DNV, there was a reduction in MHC content per half-sarcomere ($n$) in DIAm fibers expressing MHC\textsubscript{2X}. When ATP\textsubscript{iso} was normalized for this DNV-induced reduction in MHC content, ATP consumption per MHC molecule was still decreased in fibers expressing MHC\textsubscript{2X}. Following 2 wk of DNV, cross-bridge cycling rate was slower in fibers expressing MHC\textsubscript{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\textsubscript{iso} in DIAm fibers expressing MHC\textsubscript{2X}. Finally, the $\alpha_{fs}$ was unaffected by DNV. Together, these results support the conclusion that DNV reduces ATP\textsubscript{iso} in DIAm fibers expressing MHC\textsubscript{2X} by reducing MHC content and by slowing 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\textsubscript{iso}). Indeed, a decrease in ATP\textsubscript{iso} was observed, but to a greater extent than that predicted by the decrease in MHC concentration alone: ATP\textsubscript{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), hypothyroidism (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\textsubscript{2X} alone or together with MHC\textsubscript{2B}. Thus selective effects on fibers expressing MHC\textsubscript{2X} and/or MHC\textsubscript{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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