Effects of hypothyroidism on maximum specific force in rat diaphragm muscle fibers

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Geiger, Paige C., Mark J. Cody, Young Soo Han, Larry W. Hunter, Wen-Zhi Zhan, and Gary C. Sieck.
Effects of hypothyroidism on maximum specific force in rat diaphragm muscle fibers. J Appl Physiol 92: 1506–1514, 2002.—We hypothesized that 1) hypothyroidism (Hyp) decreases myosin heavy chain (MHC) content per half-sarcomere in diaphragm muscle (Dia_m) fibers, 2) Hyp decreases the maximum specific force (F_{max}) of Dia_m fibers because of the reduction in MHC content per half-sarcomere, and 3) Hyp affects MHC content per half-sarcomere and F_{max} to a greater extent in fibers expressing MHC type 2X (MHC_{2X}) and/or MHC type 2B (MHC_{2B}). Studies were performed on single Triton X-permeabilized fibers activated at pCa 4.0. MHC content per half-sarcomere was determined by densitometric analysis of SDS-polyacrylamide gels and comparison with a standard curve of known MHC concentrations. After 3 wk of Hyp, MHC content per half-sarcomere was reduced in fibers expressing MHC_{2X} and/or MHC_{2B}. On the basis of electron-microscopic analysis, this reduction in MHC content was also reflected by a decrease in myofibrillar volume density and thick filament density. Hyp decreased F_{max} across all MHC isoforms; however, the greatest decrease occurred in fibers expressing fast MHC isoforms (~40 vs. ~20% for fibers expressing slow MHC isoforms). When normalized for MHC content per half-sarcomere, force generated by Hyp fibers expressing MHC_{2A} was reduced compared with control fibers, whereas force per half-sarcomere MHC content was higher for fibers expressing MHC_{2X} and/or MHC_{2B} in the Hyp Dia_m than for controls. These results indicate that the effect of Hyp is more pronounced on fibers expressing MHC_{2X} and/or MHC_{2B} and that the reduction of F_{max} with Hyp may be at least partially attributed to a decrease in MHC content per half-sarcomere but not to changes in force per cross bridge.

thyroid hormone levels; skinned fibers; myosin heavy chain content; force per cross bridge

MYOSIN HEAVY CHAIN (MHC) isoform expression forms the basis of fiber type classification in skeletal muscle, and contractile characteristics of skeletal muscle are correlated with MHC isoform expression (3, 18, 40, 43, 44). Thyroid hormone has been shown to modulate MHC gene expression and isoform composition in adult skeletal muscle fibers (10, 13, 22, 29, 30, 32, 34, 51). However, the effects of thyroid hormone in the rat diaphragm muscle (Dia_m) are controversial. For example, previous studies have shown that hypothyroidism (Hyp) has no effect on the rat Dia_m (29), whereas others have shown an increase in the relative number of type I Dia_m fibers and a decrease in Dia_m oxidative enzyme capacity (28). Previous results from our laboratory demonstrated changes in relative MHC isoform composition, a decrease in maximum specific force (F_{max}), and a slowing of maximum shortening velocity in adult Dia_m bundles after 3 wk of Hyp (22).

Studies from our laboratory and others indicate that the F_{max} of single fibers differs with MHC isoform expression (3, 18, 19, 43, 44). Dia_m fibers expressing MHC type 2X (MHC_{2X}) alone or with MHC type 2B (MHC_{2B}) generate greater F_{max} than do fibers expressing MHC type 2A (MHC_{2A}) or the MHC slow isoform (MHC_{slow}). When force generated by rat Dia_m fibers was normalized for MHC content per half-sarcomere, no differences in F_{max} were found across fibers expressing fast MHC isoforms (18). However, Dia_m fibers expressing MHC_{slow} produced less force per half-sarcomere MHC content than fibers expressing fast MHC isoforms. Because MHC content per half-sarcomere reflects the number of cross bridges in parallel, these results indicated that the average force per cross bridge was greater in Dia_m fibers expressing fast MHC isoforms.

Previous studies in the rat Dia_m demonstrated changes in MHC content per half-sarcomere and F_{max} after unilateral denervation and during early postnatal development. After 2 wk of unilateral denervation of the rat Dia_m, we found that MHC content per half-sarcomere and F_{max} are reduced primarily in fibers expressing MHC_{2X} and MHC_{2B} (16). Similarly, we found that, during early postnatal development of the rat Dia_m, MHC content per half-sarcomere and F_{max} progressively increase and that, across all ages, MHC content per half-sarcomere and F_{max} are significantly greater in fibers expressing MHC_{2X} and MHC_{2B} than in fibers expressing MHC_{2A} and MHC_{slow} (17).

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In the present study, we hypothesized that 1) Hyp decreases MHC content per half-sarcomere in DiÌ"m fibers, 2) Hyp decreases Fmax of DiÌ"m fibers because of the reduction in MHC content per half-sarcomere, and 3) Hyp affects MHC content per half-sarcomere and Fmax to a greater extent in fibers expressing MHC2X and/or MHC2B isoforms.

**METHODS**

Twenty adult male Sprague-Dawley rats (body weight ~300 g) were assigned to control (Ctl, n = 10) or Hyp (n = 10) groups. Hyp was induced by addition of propylthiouracil to the drinking water, with a final concentration of 0.05%, for 3 wk. This 3-wk time period was previously used in our laboratory and was sufficient to cause significant alterations in MHC isoform expression and reductions in Fmax. However, this time period was not associated with any significant changes in animal body weight, a parameter that could confound the results of the study.

Animals were housed in separate cages under a 12:12-h light-dark cycle; Purina rat chow and water were provided ad libitum. At the end of the experimental period, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Blood (3 ml) was removed from each animal by intracardiac puncture. After the serum was separated, it was transferred to a microcentrifuge vial and frozen at ~80°C for later analysis of serum T4 levels. The Institutional Animal Care and Use Committee of the Mayo Clinic approved all procedures. The Institutional Animal Care and Use Committee of the Mayo Clinic approved all procedures.

**Tissue preparation and single-fiber dissection.** The DiÌ"m was removed and cut into small muscle bundles while in a mammalian Ringer solution aerated with 95% O2-5% CO2 (PO2 400–600 Torr, PCO2 35–40 Torr, pH 7.35–7.4). Muscle fiber bundles were stretched to optimal length and pinned on cork. Muscle bundles were then placed in a relaxing solution for 24 h; the relaxing solution consisted of 59.0 mM potassium acetate, 6.7 mM magnesium acetate, 5.6 mM NaATP, 10 mM EGTA, 2.0 mM dithiothreitol, 15.0 mM creatine phosphate, 1 mg/ml phosphocreatine kinase, and 50 mM imidazole for a total ionic strength of 200 mM at pH 7.0 at 5°C. Fiber bundles were stored for up to 3 wk in relaxing solution containing 50% (vol/vol) glycerol. A relaxing solution containing 1% Triton X-100 was used to permeabilize the plasma membrane. Single fibers were dissected under a dissecting microscope while in skinning solution (~20 min) and then transferred to a relaxing solution (pCa 9.0) before force measurement.

**Single-fiber mechanical measurements.** Free ionic Ca2+ concentration in the activating and relaxing solutions used for force and stiffness measurements was determined with the computer program described by Fabiato and Fabiato (15), with stability constants listed by Gerd and Lindley (20). The solutions contained 10.0 mM EGTA, 1.0 mM free Mg2+, 5.0 mM MgATP, 15.0 mM creatine phosphate, 50.0 mM imidazole, 2.0 mM dithiothreitol, and phosphocreatine kinase (1 mg/ml), with a total ionic strength of 150 mM. pCa of the relaxing and activating solutions were 9.0 and 4.0, respectively. All mechanical measurements were made while fibers were maintained at 15°C.

Small stainless steel hooks were used to attach dissected fibers between a force transducer (model AE-801, Akron, Ohio), with a resonant frequency of 5 kHz, and a servo-motor (model G120DT, General Scanning), with a step time of 800 μs. Fiber ends were fixed in 5% glutaraldehyde and anchored using aluminum foil T clips to maintain noncompliant attachments of fibers to the force transducer and servo-controlled motor. The attached fiber was positioned horizontally in a temperature-controlled flow-through acrylic chamber (volume 120 μl) on the stage of an inverted microscope (model IMT-2, Olympus). Sarcomere length (set at 2.5 μm) was monitored with first-order laser diffraction (He-Ne laser; model LSC 30D, UDT Sensors). During experiments, sarcomere length was stabilized with the Brenner cycling method (7) as modified by Sweeney et al. (48). A data acquisition board and LabView-based software were used to record signals. Muscle fiber length (~2.0 mm) was measured using a reticle in the microscope eyepiece (×10 Olympus Plan 10, 0.30 numerical aperture (NA)). A ×40 objective (Olympus LWD CD Plan 40, 0.55 NA) was used to measure the xy fiber diameter. This objective was also used to measure the z fiber diameter (depth) by zeroing the microscope fine focus control wheel facing on the top of the fiber and focusing through to the bottom of the fiber. Previous measurements revealed a 20% error in the depth measurement using this method compared with direct measurement of the fiber z-axis using confocal microscopy (18). Therefore, fiber cross-sectional area was calculated directly from fiber width (xy) and depth (zz) using an established correction factor for z-axis distortion. Fiber cross-sectional area was measured while the fiber was mounted on the stage of an inverted microscope at a sarcomere length of 2.5 μm.

Fibers were perfused with a pCa 9.0 solution for the measurement of baseline force. To verify that force returned to its original baseline level after maximal activation in pCa 4.0 solution, the fiber was again perfused with a pCa 9.0 solution. Fmax (N/cm²) was determined by dividing the iso- metric force generated at pCa 4.0 by fiber cross-sectional area. Fmax was also divided by the estimated value of MHC content per half-sarcomere (see MHC content per half-sarcomere measurements) to determine the force per half-sarcomere MHC content (N/μg MHC content).

Muscle fiber stiffness was determined from sinusoidal length oscillations (0.2% of optimal length) at 2 kHz during activation at pCa 4.0 in the presence and absence (rigor solution) of ATP. Stiffness measurements in pCa 4.0 and rigor were normalized for fiber cross-sectional area. It was assumed that fiber stiffness during rigor reflects full recruitment of all available cross bridges. The ratio of fiber stiffness in rigor solution to that in pCa 4.0 solution (with ATP) thus reflected the fraction of cross bridges in the strongly bound force-generating state (6).

**MHC content per half-sarcomere measurements.** MHC concentration in rat DiÌ"m single fibers was determined as previously described (18). To determine fiber volume and the number of sarcomeres in a given fiber length, single fibers were fixed in 4% paraformaldehyde for 30 s and imaged using a microscope (Optiphot-2, Nikon; ×20, 0.5-NA objective) with a charge-coupled device camera (model 72, MTI). From this projected image, the number of sarcomeres in series was counted, and width and depth measurements were used to determine fiber cross-sectional area. Fiber cross-sectional area was normalized to a sarcomere length of 2.5 μm, the sarcomere length at which force was measured. The number of sarcomeres in series and the fiber volume measurements were used to determine the volume of a half-sarcomere.

After these measurements, 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) bromphenol blue at pH 6.8 and denatured by boiling for 2 min. A modification of the procedure of Sugura and Murakami (47) was used to prepare the gradient gels. The stacking gel contained 3.5% acrylamide (pH 6.8), and the separating gel contained 5–8% acrylamide (pH 8.8) with 25% glycerol (8 × 10 cm, 0.75 mm thick; SE250, Hoefer). Control samples of Dia_m bundles in a 1:200 dilution of SDS sample buffer (–9.0 ng/µl MHC concentration determined by the Bradford method (4) were used to compare migration patterns of the MHC isoforms. Sample volumes of 10 µl were loaded per lane. The gels were silver stained according to the procedure described by Oakley et al. (35).

Identification of MHC isoforms by migration patterns was confirmed by Western blot analysis, as previously described (18, 19). One of the following mouse monoclonal or polyclonal antibodies was used to identify MHC isoform expression: NCL (IgG; Novocastra, which reacts with MHCslow; SC.71 (IgG; American Type Culture Collection), which reacts with MHCh, BF-F3 (IgM; Schiaffino), which reacts with MHCh, and BJ (IgG; Schiaffino), which reacts with all but the MHCslow isoform. Isoform specificity of these antibodies was previously determined (26, 41). A biotinylated secondary antibody specific to IgG (SC.71 and BF-35; NCL) or IgM (BF-F3) was used, and bands were visualized with alkaline phosphatase (Vectastain ABC kit, Vector Labs).

A standard curve of known concentrations of purified rabbit MHC (M-3889, Sigma) was run on every gel to determine MHC concentration in rat Dia_m fibers. The Bradford method (4) was used to verify the standard concentrations of MHC run on the gels. This technique has been previously described (18). After silver staining, a high-resolution scanner (600 dpi; Microtek ScanMaker 5) was used to image the gels. Background staining was subtracted from the density of the electrophoretic bands to determine the brightness-area product for each Dia_m fiber. The relationship between the brightness-area product and MHC concentration was linear across a range from 0.01 to 0.25 µg/µl. The MHC concentration of the single fiber was determined from the standard curve and was multiplied by the half-sarcomere volume of the fiber to determine MHC content per half-sarcomere.

Electron-microscopic imaging of single muscle fibers. Single Dia_m fibers were dissected, and a small segment (1–2 mm) of each fiber was analyzed for MHC isoform content, as described above. The remaining portion of each fiber was pinned to a flat Teflon strip and then immersion fixed overnight at 4°C in 2 ml of Trump's fixative. Subsequently, processing of single fibers for electron microscopy was performed at room temperature, except where specified. Fibers were rinsed for 30 min in two changes of 0.1 M phosphate buffer, pH 7.2, and then postfixed for 5 min in phosphate-buffered 1% OsO_4. After they were rinsed for 10 min in two changes of distilled water, each fiber was stained with 2% uranyl acetate for 20 min at 60°C. Each fiber was then rinsed in water for 10 min, dehydrated in graded ethanol to 100%, cleared in propylene oxide, and infiltrated with Spurr's resin. Each fiber was then unpinned, placed in a small drop of resin on a microscope slide, and cut transversely in the center. The slide was then placed on the stage of a light microscope, and sarcomere length was determined. The average length of 10 contiguous sarcomeres closest to each cut end was measured in eight different myofibrils. The measurements were averaged to yield the mean sarcomere length for each fiber. The two fiber sections were then placed in resin in a chen mold, oriented parallel to each other with the cut ends together, and polymerized overnight at 60°C. Thin (80-nm) cross sections were cut on a Reichert Ultracut E ultramicrotome, placed on 200-mesh copper grids, and poststained with lead citrate. Sections were imaged on an electron microscope (model 1200 EXII, JEOL) operating at 60 kV. Electron micrographs were taken for morphological examination of fibers; each electron micrograph was saved as a digital image of 1,024 × 1,024 pixels.

Ultrastructural analysis of single fibers. The myofibrillar volume density (i.e., the percentage of fiber volume occupied by myofibrils) of Dia_m fibers from Ctl and Hyp rats was determined from electron micrographs taken at ×15,000. Each electron micrograph was overlaid with a transparent square grid of 225 points. To estimate myofibrillar volume density, the number of points coincident with myofibrils was counted and expressed as the percentage of total points. Twelve randomly selected electron micrographs of each fiber were examined, and the data were averaged to yield one value.

Within a myofibril, thick filament density (number of myofilaments per unit area) was determined from electron micrographs taken at ×75,000. In this case, each micrograph was overlaid with a transparent square grid, with each square encompassing an area of 0.04 µm². The number of thick filaments within selected squares was counted following Gundersen's rules of sampling (24), and thick filament density was expressed as the number of myofilaments per square micrometer. For each fiber, thick filament density was determined in 12 myofibrils, and values were averaged to provide a single estimate.

Statistical analysis. Comparison of fiber cross-sectional area, Fmax, MHC content per half-sarcomere, force per half-sarcomere MHC content, and the fraction of cross bridges in the force-generating state across fibers expressing different MHC isoforms and between Ctl and Hyp fibers was done by two-way ANOVA. A Student's t-test with Bonferroni's correction was used as a post hoc analysis for comparison between fiber types when appropriate. Values are means ± SE. A power analysis was performed for each parameter to determine the minimal change from control values that could be detected using the number of animals per experimental group (n = 10) at a β-level of 0.8. Statistical significance was indicated by P < 0.05.

RESULTS

Body weight. At the beginning of the study, body weights of Ctl and Hyp animals were 320 ± 5 and 309 ± 3 g, respectively. During the experimental period, there were no significant changes in body weight. Final body weights for the Ctl and Hyp groups were 330 ± 8 and 296 ± 4 g, respectively, after the 3-wk experimental period.

T3 and T4 serum levels. Serum T3 and T4 levels were measured on a subset of Hyp and Ctl animals (n = 5 per group). Serum T3 averaged 51.3 ng/dl and serum T4 averaged 4.8 mg/dl in the Ctl group. However, serum T3 and T4 values were below detectable levels in the Hyp group.

MHC isoform expression. In the present study, MHC isoform expression was determined by SDS-PAGE and Western blot analysis in 223 Dia_m fibers (n = 77 Ctl and n = 146 Hyp). The adult rat Dia_m is a mixed muscle expressing four MHC isoforms (Fig. 1, Table 1). The majority of control Dia_m single fibers (n = 57) sampled in the present study expressed a single MHC isoform. Of these fibers, 25 (33%) expressed MHCslow,
15 (19%) expressed MHC2A, and 17 (22%) expressed MHC2X. Coexpression of MHC2X and MHC2B was detected in 20 (26%) of the Ctrl fibers, and singular expression of MHC2B was not detected in any of the Ctrl fibers. These results are in general agreement with previous findings in our laboratory for the rat Dia\textsubscript{m} (45). These results should not be interpreted as characterizing the fiber type distribution of the Dia\textsubscript{m} since our sampling procedure was biased. Fibers expressing MHC2\textsubscript{A}a and MHC\textsubscript{slow} were often more difficult to dissect because of their smaller size and fragility. Therefore, to obtain a sufficient number of these fibers for statistical analysis, dissection was often selective.

After 3 wk of Hyp, MHC isoform coexpression within single Dia\textsubscript{m} fibers was far more prevalent, although it should be pointed out that a systematic characterization of fiber type distribution was not performed. Similar to control, in 63 of the 146 Hyp Dia\textsubscript{m} fibers sampled, singular expression of MHC\textsubscript{slow} (n = 25), MHC2\textsubscript{A} (n = 11), and MHC2\textsubscript{X} (n = 27) was still detected, and singular expression of MHC2B was not. Among the remaining 83 Hyp fibers, nine different combinations of MHC isoform coexpression were observed (Table 1).

For comparison of single-fiber cross-sectional area, MHC content, and F\textsubscript{max}, fibers were grouped according to the predominant MHC isoform expressed (>40%). This classification scheme resulted in four major fiber groups: MHC\textsubscript{slow}, MHC2\textsubscript{A}, MHC2\textsubscript{X}, and MHC2B. All the fibers classified as predominantly expressing MHC2B coexpressed MHC2X. Therefore, the fourth fiber type classification was termed MHC2B/2X for comparison with Ctrl Dia\textsubscript{m} fibers coexpressing MHC2B and MHC2X isoforms.

Cross-sectional area. In Ctrl Dia\textsubscript{m} fibers, the cross-sectional area of fibers expressing MHC2X and/or MHC2B was greater than that of fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} (Fig. 2A; P < 0.05). In addition, in the Ctrl Dia\textsubscript{m}, the cross-sectional area of fibers coexpressing MHC2B and MHC2X was significantly greater than that of fibers expressing MHC2X alone (Fig. 2A; P < 0.05). No significant difference in cross-sectional area between fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} was found in Ctrl Dia\textsubscript{m} fibers. After Hyp, the cross-sectional area of fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} increased compared with Ctrl (Fig. 2A; P < 0.05). The cross-sectional area of fibers expressing MHC2X and/or MHC2B in the Hyp Dia\textsubscript{m} was not significantly different from Ctrl (Fig. 2A). However, the cross-sectional area of Hyp fibers expressing MHC2X and/or MHC2B remained significantly greater than that of fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow}.

MHC content per half-sarcomere. In Ctrl Dia\textsubscript{m} fibers expressing MHC2X alone or MHC2X in combination with MHC2B had the greatest MHC content per half-sarcomere, approximately threefold higher than that of fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} (Fig. 2B; P < 0.05). No significant difference in MHC content per half-sarcomere was found between Ctrl fibers expressing MHC\textsubscript{slow} and MHC2\textsubscript{A} isoforms. After Hyp, MHC content per half-sarcomere of Dia\textsubscript{m} fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} was not significantly changed (Fig. 2B). However, in the Hyp Dia\textsubscript{m}, the MHC content per half-sarcomere of Dia\textsubscript{m} fibers expressing MHC2\textsubscript{X}, alone or in combination with MHC2B, decreased by ~50% compared with Ctrl fibers (Fig. 2B; P < 0.05). As a result, there were no fiber type differences in MHC content per half-sarcomere in the Hyp Dia\textsubscript{m}.

Electron-microscopic analysis. Myofibrillar volume density and thick filament density of Dia\textsubscript{m} fibers expressing MHC2\textsubscript{A} and MHC\textsubscript{slow} did not appear to be different between Ctrl and Hyp animals. However, even qualitative inspections suggested differences in myofibrillar volume density (Fig. 3) and thick filament density (Fig. 4) for Dia\textsubscript{m} fibers expressing MHC2X and/or MHC2B between Ctrl and Hyp animals. Therefore, because these were the only fibers that displayed differ-

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Table 1. MHC isoform expression in Ctrl and Hyp Dia\textsubscript{m} fibers

<table>
<thead>
<tr>
<th>Predominant MHC Isoform Expression</th>
<th>n</th>
<th>MHC\textsubscript{slow}</th>
<th>MHC2\textsubscript{A}</th>
<th>MHC2\textsubscript{X}</th>
<th>MHC2\textsubscript{B}</th>
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Values are means ± SE; n, number of single fibers. %Expression of myosin heavy chain (MHC) isoforms is based on relative densitometric analysis of MHC bands in SDS-PAGE. Ctl, control; Hyp, hypothyroid (3 wk); Dia\textsubscript{m}, diaphragm muscle.

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Fig. 1. Myosin heavy chain (MHC) isoform expression within the rat diaphragm muscle (Dia\textsubscript{m}) identified by SDS-PAGE. Lane 1 is a mixed control (Ctl) Dia\textsubscript{m} bundle; other lanes represent hypothyroid (Hyp) single fibers expressing different MHC isoforms (MHC2\textsubscript{A}, MHC2\textsubscript{X}, MHC2\textsubscript{B}, MHC\textsubscript{slow}).
filament density was directly proportional to sarcomere length ($r^2 = 0.96$ in Ctl and Hyp). Therefore, thick filament density in myofibrils was compared only after normalization for a standard sarcomere length of 2.7 μm (approximate optimal sarcomere length for force generation). The average normalized thick filament density in Ctl Diam fibers expressing MHC2X and/or MHC2B was 1,467 ± 48 filaments/μm² compared with 1,212 ± 31 filaments/μm² in Hyp fibers expressing these isoforms ($P < 0.05$). Considering the reduced myofibrillar volume density and lower thick filament density, there was an estimated 22% overall decrease in thick filaments in Diam fibers expressing MHC2X and/or MHC2B in Hyp rats compared with Ctl.

$F_{max}$ In Ctl Diam, $F_{max}$ differed across all MHC isoforms in the following order: MHC2B/2X > MHC2X > MHC2A > MHCslow (Fig. 5A; $P < 0.05$). After Hyp, a significant decrease in $F_{max}$ was found for all Diam fibers compared with Ctl (Fig. 5A; $P < 0.05$). However, the $F_{max}$ of Hyp fibers expressing fast MHC isoforms decreased to the greatest extent (~40%) compared with a decrease of ~20% in fibers expressing MHCslow. Despite the dramatic decrease in $F_{max}$ in fibers expressing MHC2X and/or MHC2B, $F_{max}$ was still significantly lower in fibers expressing MHC2A and MHCslow in the Hyp Diam (Fig. 5A; $P < 0.05$).

**Fraction of cross bridges in the force-generating state.**
To evaluate a possible change in the recruitment of cross bridges after Hyp, the ratio of fiber stiffness during activation in pCa 4.0 solution to that during activation in rigor solution (pCa 4.0 without ATP) was used as an estimate of the fraction of cross bridges in the force-generating state (Fig. 5C). No significant differences in the fraction of cross bridges in the force-generating state were found across fibers expressing different MHC isoforms in Ctl or Hyp Diam. In addition, after Hyp, there was no change in the fraction of cross bridges in the force-generating state compared with Ctl fibers.

**DISCUSSION**

The results of the present study support our hypothesis that, in rat Diam fibers, Hyp induces a decrease in MHC content per half-sarcomere (the number of cross

Fig. 2. A: cross-sectional area (CSA) measurements of Ctl and Hyp rat Diam fibers expressing different MHC isoforms. Values are means ± SE. B: MHC content per half-sarcomere in Ctl and Hyp rat Diam fibers expressing different MHC isoforms. Values are means ± SE.

Figures in MHC content per half-sarcomere, we performed a more rigorous stereological analysis of myofibrillar volume density and thick filament density in a subset of fibers expressing MHC2X and/or MHC2B. In these fibers, myofibrillar volume density was 74.0 ± 0.6% in Ctl ($n = 5$) compared with 69.5 ± 1.4% in Hyp ($n = 5$) rats (Fig. 3; $P < 0.05$). The volume density of other extramyofibrillar compartments was not systematically assessed; however, mitochondria and, to a much lesser extent, sarcoplasmic reticulum and T tubules occupied most of this space. Regression analysis indicated that myofibrillar volume density was not correlated to sarcomere length ($r^2 = 0.04$ and 0.14 in Ctl and Hyp, respectively).

Ctl and Hyp displayed fairly normal thick-thin filament lattice structure, but in Hyp, Diam fibers expressing MHC2X and/or MHC2B thick filaments appeared to be less densely packed than in Ctl (Fig. 4). Consistent with previous reports (5, 38), we found that thick
bridges in parallel). It also appears that the reduction in MHC content per half-sarcomere may, at least in part, account for the Hyp-induced decrease in Fmax of Diaₐ fibers expressing MHC2X and/or MHC2B isoforms. Electron-microscopic analysis also indicated a reduction in thick filament density in fibers expressing MHC2X and/or MHC2B. Although the decrease in MHC content per half-sarcomere was consistent with the reduction in Fmax that was observed after Hyp, normalization of force for MHC content per half-sarcomere indicated a possible effect of Hyp on the unitary force generated per cross bridge. Thus the present results suggest that Hyp results in MHC isoform-specific adaptations in the rat Diaₐ with an effect on the number of cross bridges in parallel and the force-generating capacity of individual cross bridges.

Effects of Hyp on MHC isoform expression. Gene regulation of MHC isoform expression was affected by 3 wk of Hyp, as evidenced by the significant polymorphism of MHC isoform expression in single Diaₐ fibers. The effect of Hyp on rat Diaₐ MHC isoform expression was previously examined in whole tissue, and thus coexpression of MHC isoforms within single fibers was not assessed (22). Therefore, the results of the present study provide important new information regarding the relative coexpression of MHC isoforms in single Diaₐ fibers after Hyp. The significant polymorphism of MHC isoform expression reported in the present study is in agreement with previous results in the plantaris muscle of rats after Hyp (11, 14). These investigators identified 13 different combinations of MHC isoform coexpression in the plantaris muscle after Hyp, and only 33% of the fibers sampled expressed a single MHC isoform (11). Similar to the Diaₐ, the plantaris muscle expresses all four adult MHC isoforms, and the significant polymorphism seen in the Diaₐ and the plantaris muscle may be a unique adaptation of mixed-fiber type muscles to Hyp.

The presence of Diaₐ fibers coexpressing unique combinations of MHC isoforms (e.g., coexpression of MHCslow, MHC2X, and MHC2B in the absence of MHC2A expression) does not support a systematic continuum of MHC transitions, e.g., MHCslow→MHC2A→MHC2X→MHC2B, as proposed by Pette and colleagues (31, 36, 46, 50). Fibers coexpressing these unique combinations of MHC isoforms have been termed “jump fibers,” and, in addition to being present in limb muscles of Hyp rats, they have also been observed after
hindlimb suspension and exposure to microgravity (8, 9, 49). Although such jump fibers were found in the rat Dia_m after Hyp, the prevalent patterns of MHC isoform coexpression observed in the Hyp Dia_m were consistent with the continuum theory of Pette and colleagues (31, 36, 46, 50). This was also true for the patterns of MHC isoform coexpression reported for hindlimb muscles after Hyp, hindlimb suspension, and microgravity exposure (8, 9, 49). Yet, the presence of jump fibers indicates that an inflexible continuum of MHC isoform transition does not exist.

In addition to transitions in MHC isoform expression in single rat Dia_m fibers, Hyp was also associated with a reduction in MHC content per half-sarcomere in Dia_m fibers expressing MHC2X and/or MHC2B isoforms. This is the first report of changes in myosin content, or even myofibrillar protein content, in single muscle fibers after Hyp treatment. Most previous studies have determined only the relative changes in MHC isoform composition of muscles after Hyp treatment (1, 2, 8, 9, 11, 14). However, one study did report a reduction in myofibrillar protein content in the rat soleus and planataris muscles after Hyp treatment (12). Yet, when myofibrillar content was normalized for changes in muscle weight in this study, no significant difference in myofibrillar content was found compared with Ctl muscles. Single-fiber analysis of MHC content per half-sarcomere in the present study eliminated confounding factors such as changes in muscle weight and noncontractile material (i.e., connective tissue protein or interstitial fluid volume) and allowed direct quantitation of the effects of Hyp on the contractile machinery itself.

Previous studies in limb skeletal muscle have indicated that the effects of Hyp are muscle specific (29). For example, in the soleus muscle, Hyp increased expression of MHCslow mRNA, whereas in a fast-twitch muscle predominantly expressing MHC2B, Hyp had a lesser effect and slightly increased the expression of MHC2A (29).

The effect of Hyp may also depend on muscle load and/or activation history. In a recent study, Caiozzo and colleagues (11) reported that the response of skeletal muscle to altered thyroid state is dependent on the loading state of the muscle. In this respect, it should be recognized that the Dia_m is not a load-bearing muscle, and it is further distinguished by a unique activation history compared with limb muscles. The duty cycle (proportion of time active vs. inactive) of the Dia_m is very high compared with that of limb muscles (~40% compared with ~2% for the extensor digitorum longus muscle and ~14% for the soleus muscle) (25, 42). Therefore, the effects of Hyp in limb muscles cannot be directly extrapolated to the Dia_m. Clearly, the effect of Hyp on MHC isoform expression appears to be muscle specific, and the results of the present study indicate that the effects of Hyp in the rat Dia_m are MHC isoform specific, with a greater loss of MHC content in fibers expressing fast MHC isoforms.

**Effects of Hyp on force generation.** F_max decreased across all MHC isoforms after Hyp. This is in agreement with previous results from our laboratory demonstrating a decrease in F_max in rat Dia_m strips with Hyp (22). When F_max was normalized for MHC content per half-sarcomere in Ctl rat Dia_m fibers, differences in force generation across fibers expressing fast MHC isoforms were eliminated. In contrast, Ctl Dia_m fibers expressing MHCslow generated less force than fibers...
expressing fast MHC isoforms, even after normalization for MHC content per half-sarcomere. These results are consistent with previous results from our laboratory (18) and suggest that, in the Ctl rat Dia3m, force per cross bridge is comparable across fibers expressing fast MHC isoforms but lower in fibers expressing MHCslow. After 3 wk of Hyp, Fmax normalized for MHC content per half-sarcomere was unaffected in fibers expressing MHCslow and decreased in fibers expressing MHC2A. In contrast, force per half-sarcomere MHC content increased in fibers expressing MHC2X and/or MHC2B. Thus the force per cross bridge appeared to be differentially affected by Hyp. These results clearly indicate that something other than a reduction in the number of available cross bridges (MHC content per half-sarcomere) is causing the reduction of specific force induced by Hyp.

Permeabilization of muscle fibers may result in alterations in lattice spacing due to changing fiber volume (21). When the filament lattice is compressed below its in situ value, movement of the S1 fragment of the cross bridge is hindered, resulting in force inhibition (33). Electron-microscopic analysis suggested that filament lattice spacing was not affected by Hyp in Dia3m fibers expressing MHCslow and MHC2A. However, in Hyp fibers expressing MHC2X and/or MHC2B, myofibrillar volume density and thick filament density were reduced. If anything, such a decrease in thick filaments would increase the lateral spacing between thick and thin filaments, and this may have affected the probability of cross-bridge attachment and, thus, the average unitary force generated per cross bridge. Yet, it seems improbable that such an alteration in filament lattice spacing could have so markedly increased the average force per cross bridge in fibers expressing MHC2X and/or MHC2B.

Huxley’s original two-state model of cross-bridge cycling involves two distinct cross-bridge states: a force-generating state, in which cross bridges are strongly bound to actin, and a non-force-generating state, in which cross bridges are detached from actin (27). It could be argued that slower cross-bridge cycling kinetics would produce a longer duty cycle for cross-bridge attachment, resulting in a higher fraction of strongly bound cross bridges and, therefore, a greater Fmax (23, 39). To consider this possibility, the fraction of strongly bound cross bridges was estimated from muscle fiber stiffness measurements in the present study. However, the fraction of strongly bound cross bridges did not change after Hyp and, therefore, could not account for changes in Fmax or force per MHC content in the present study. These results agree with previous studies from our laboratory demonstrating no significant difference in the fraction of strongly bound cross bridges across fibers expressing different MHC isoforms (18, 44), despite an approximately twofold difference in cross-bridge cycling kinetics (as estimated by the rate of force redevelopment after rapid release and restretch) (44). In addition, we did not see a significant difference in the fraction of strongly bound cross bridges after 2 wk of unilateral denervation, a condition that also results in reduced Fmax (16) and decreased cross-bridge cycling kinetics (unpublished observations). On the basis of these results, it seems unlikely that differences in cross-bridge cycling kinetics can explain the differences in Fmax generation after Hyp in rat Dia3m fibers.

In summary, we conclude that Hyp results in MHC isoform-specific adaptations in the rat Dia3m, with an effect on MHC content per half-sarcomere (the number of cross bridges in parallel) and the force-generating capacity of individual cross bridges. However, the decrease in Fmax of Dia3m fibers induced by Hyp cannot be solely attributed to a reduction in MHC content per half-sarcomere or to changes in the average force per cross bridge.

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