Plasticity in Skeletal, Cardiac, and Smooth Muscle

Selected Contribution: Mechanisms underlying increased force generation by rat diaphragm muscle fibers during development

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Selected Contribution: Mechanisms underlying increased force generation by rat diaphragm muscle fibers during development. J Appl Physiol 90: 380–388, 2001.—It has been found that maximum specific force (Fmax; force per cross-sectional area) of rat diaphragm muscle doubles from birth to 84 days (adult). We hypothesize that this developmental change in Fmax reflects an increase in myosin heavy chain (MHC) content per half-sarcomere (an estimate of the number of cross bridges in parallel) and/or a greater force per cross bridge in fibers expressing fast MHC isoforms compared with slow and neonatal MHC isoforms (MHCslow and MHCneo, respectively). Single Triton 100-X-permeabilized fibers were activated at a pCa of 4.0. MHC isoform expression was determined by SDS-PAGE. MHC content per half-sarcomere was determined by densitometric analysis and comparison to a standard curve of known MHC concentrations. MHC content per half-sarcomere progressively increased during early postnatal development. When normalized for MHC content per half-sarcomere, fibers expressing MHCslow and coexpressing MHCneo produced less force than fibers expressing fast MHC isoforms. We conclude that lower force per cross bridge in fibers expressing MHCslow and MHCneo contributes to the lower Fmax seen in early postnatal development.

postnatal development; maximum specific force; myosin heavy chain content; force per cross bridge; single fibers

IN THE RAT DIAPHRAGM MUSCLE (Diam), early postnatal development is characterized by dramatic transitions in myosin heavy chain (MHC) isoform expression. During the first 3 postnatal wk, expression of the neonatal MHC isoform (MHCneo) decreases, whereas expression of MHC2X and MHC2B isoforms appears only after the second postnatal week and increases thereafter (18–20, 35, 44–47). This postnatal transition from MHCneo to adult fast MHC isoform expression was found to be associated with changes in contractile properties of the rat Diam (18, 31, 35, 44, 46, 47). In particular, it was noted that the increase in maximum specific force (Fmax; force per cross-sectional area) of the rat Diam during early postnatal development was associated with an increase in the relative expression of fast MHC isoforms (18, 35, 44, 46, 47).

Although controversial (8), previous studies in the rat Diam have indicated that fibers expressing MHC2X and MHC2B isoforms have a greater Fmax than fibers expressing the MHCslow and MHC2A isoform (6, 9, 10, 33, 34). In the adult rat Diam, differences in MHC content per half-sarcomere (reflecting the number of cross bridges in parallel) exist across fibers expressing different MHC isoforms, with fibers expressing MHC2X and MHC2B having greater MHC content per half-sarcomere than fibers expressing MHCslow and MHC2A (9). When Fmax of adult Diam fibers was normalized for MHC content per half-sarcomere, fibers expressing all fast MHC isoforms (MHC2A, MHC2X, and MHC2B) still generated greater force than fibers expressing MHCslow (9). This indicated a greater force per cross bridge in rat Diam fibers expressing fast MHC isoforms. Thus the postnatal increase in Fmax of the rat Diam could reflect the emergence of MHC2X and MHC2B expression (18, 35, 46, 47). In the present study, we examined the hypothesis that the postnatal
increase in \( \text{Dia}_m F_{\text{max}} \) reflects an increase in MHC content per half-sarcomere and/or a greater force per cross bridge in fibers expressing fast MHC isoforms compared with MHC\(_{\text{slow}} \) and MHC\(_{\text{neo}} \).

**METHODS**

Experiments were performed on male Sprague-Dawley rats at postnatal days 0, 14, and 28 (D0, D14, and D28, respectively) and on 84-day-old rats (adults) \( (n = 8 \) rats for each age group). Pregnant mothers were received at 14 days gestation, and litter size was culled to eight pups after parturition. To ensure normal body growth, pups from smaller litters were not studied. Animal body weights were measured daily. The pups were housed with their lactating mothers until D21 when they were weaned; thereafter, they were housed two per cage under a 12:12-h light-dark cycle. The adult animals and postweaning pups were fed Purina rat chow and provided with water ad libitum. The Institutional Animal Care and Use Committee of the Mayo Clinic approved all procedures.

**Tissue preparation and single-fiber dissection.** Tissue was excised intramuscularly with ketamine (60 mg/kg) and xylazine (2.5 mg/kg), and the Dia\(_m \) was excised. The Dia\(_m \) was cut into muscle bundles, stretched \( \sim 20\% \) beyond resting length, pinned on cork, and placed in a relaxing solution at 5°C consisting of 59.0 mM potassium acetate, 6.7 mM magnesium acetate, 5.6 mM NaATP, 10 mM EGTA, 2.0 mM dithiothreitol (DTT), 15.0 mM creatine phosphate (CrP), 1 mg/ml phosphocreatine kinase (PKC), and 50 mM imidazole. An ionic strength of 150 mM and a pH of 7.0 were adjusted with propionic acid. After 24 h, fiber bundles were transferred to relaxing solution containing 50% glycerol (vol/vol) and stored for up to 3 wk. On the day of the study, fiber bundles were dissected and placed into relaxing solution containing 1% Triton X-100 for 20 min to permeabilize the plasma membrane. While in this skimming solution, single fibers were dissected under a stereo microscope. Because of the technical difficulty of dissecting single fibers in the neonatal Dia\(_m \), muscle bundles \( (\sim 5–10 \) fibers) were used at D0. The single fibers or neonatal bundles were then transferred from the skimming solution to a relaxing solution (pCa 9.0).

**Single-fiber mechanical measurements.** The activating (high Ca\(^{2+} \)) and relaxing (low Ca\(^{2+} \)) solutions were prepared using the computer program described by Fabiato and Fabiato (7) with stability constants listed by Godt and Lindley (11). Solutions contained the following (in mM unless otherwise specified): 10.0 EGTA, 1.0 free Mg\(^{2+} \), 5.0 NaATP, 15.0 CrP, 50.0 imidazole, 2.0 DTT, and PCK at 1 mg/ml with a total ionic strength of 150 mM. The ionic strength and pH of 7.0 were adjusted with propionic acid. Relaxing solution had a pCa of 9.0, and the activating solution had a pCa of 4.0.

Noncompliant attachments of the fibers to a force transducer and a servo-controlled motor were maintained by fixing the fiber ends in a 5% gluteraldehyde solution. To further reduce compliance and allow for fiber mounting, aluminum foil T clips were attached at the fixed ends of the fiber. Fibers were mounted on two stainless steel hooks in a temperature-cooled flow-through acrylic chamber (volume = 120 ml) on the stage of an Olympus IMT-2 inverted microscope. One end of the fiber was attached to a servo-motor (General Scanning, G120DT) with a step time of 800 ms, and the other end of the fiber was attached to a force transducer (Aksjeselskapet, AE-801) with a resonant frequency of 5 kHz. A reticule in the eyepiece of the inverted microscope was used to measure the length \( \times 10 \) Olympus Plan 10, 0.30 numerical aperture (NA), width (xy-plane), and depth (xz-plane) of fibers \( \times 40 \) Olympus LWD CD Plan 40, 0.55 NA). The depth measurements were made by setting the microscope fine focus to zero while focusing on the top of the fiber and then focusing through the fiber to the bottom. In a previous study, we obtained a correction factor to account for z-axis distortion due to the optics used (9). First-order laser diffraction (He-Ne laser, UDT Sensors, LSC 30D) was used to set the sarcomere length to 2.5 \( \mu \)m, the optimal length \( (L_0) \) for force development. Sarcomere length was stabilized during experiments with Brenner cycling (4) as modified by Sweeney et al. (40). Force signals were digitized at 1 kHz using a Lab-View data-acquisition board (National Instruments).

Initially, fibers were perfused with a pCa 9.0 solution to obtain a baseline force measurement. While the fiber was kept in the same location, the perfusate was switched to a pCa 4.0 solution to maximally activate fibers. Fibers were exposed to the pCa 4.0 solution until maximum force was stable, and fibers were then reexposed to the pCa 9.0 solution to verify that force returned to the original baseline level. \( F_{\text{max}} \) (N/cm\(^2\)) was calculated by dividing the maximum isometric force during pCa 4.0 activation by the corrected fiber cross-sectional area. Force per half-sarcomere MHC content \( (N/\mu g \text{ MHC} \text{ content}) \) was obtained by dividing the maximum isometric force by MHC content per half-sarcomere (see below).

Muscle fiber stiffness was determined from sinusoidal length oscillations (0.2% \( L_0 \)) at 2 kHz, during activation at pCa 4.0 in the presence and absence (rigor solution) of ATP. Stiffness measurements under both conditions were normalized for fiber cross-sectional area. Rigor stiffness was assumed to reflect full recruitment of all available cross bridges. Thus the fraction of cross bridges in the strongly bound force-generating state was estimated from the ratio of fiber stiffness during rigor solution compared with activation at pCa 4.0 (with ATP) (3).

**Determination of MHC content per half-sarcomere.** MHC content per half-sarcomere in single rat Dia\(_m \) fibers was determined as previously described (9). Briefly, the first step in determining MHC content per half-sarcomere involved an accurate determination of the volume of each fiber segment. Single fibers or neonatal bundles \( (\sim 1.5–2.5 \text{ mm in length}) \) were fixed in 4% paraformaldehyde for 30 s and then placed on a microscope stage (Nikon Optiphot-2) with a MTI CCD72 camera. With the use of a Nikon Plan \( \times 20 \) lens (0.5 NA), a fiber image was projected onto a video screen. From this projected image, the number of sarcomeres in series was counted and fiber cross-sectional area was determined from width and depth measurements. Because force measurements were obtained at a sarcomere length of 2.5 \( \mu \)m, fiber cross-sectional area was normalized to this sarcomere length. This was accomplished by measuring the sarcomere length in the fixed fiber segment, dividing this by 2.5 \( \mu \)m, and multiplying this ratio by the fiber cross-sectional area. From the number of sarcomeres in series and the fiber volume measurements, the volume of a half-sarcomere was determined.

Fibers were then transferred to 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. To denature the fibers, samples were boiled for 2 min. A modification of the procedure by Sugiyama and Murakami (39) was used to prepare gradient gels. The separating gel contained 5–8% acrylamide (pH 8.8) with 25% glycerol, and the stacking gel contained 3.5% acrylamide (pH 6.8) (8 \( \times \) 10 cm, 0.75 mm thick, Hoefer SE250). Control samples of Dia\(_m \) bundles were loaded at 1:200 dilution of SDS samples buffer \( [\sim 9.0 \text{ ng/} \mu \text{M HC} \text{ content} \] \) determined by Bradford method (2) to compare migra-
FORCE GENERATION IN DEVELOPING RAT DIAPHRAGM FIBERS

Table 1. MHC isoform coexpression in the rat diaphragm muscle at day 14 postnatal development

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<th>Predominant MHC Isoform Expression</th>
<th>Percent Expression</th>
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Values are means ± SE; n, no. of single fibers. %Expression of myosin heavy chain (MHC) isoforms is based on relative densitometric analysis of MHC bands in SDS-PAGE. Neo, neonatal.

RESULTS

MHC isoform expression. In the present study, MHC isoform expression was determined in 16 neonatal bundles and 127 single fibers by SDS-PAGE and Western blot analysis (Fig. 1). At D0, the MHC2X, MHCslow, and MHC2A isoforms were all expressed but in different proportions. The MHC isoform composition of single Dia m fibers could not be determined directly at D0 because of the technical difficulty of dissecting the extremely small and fragile fibers at this age. However, immunohistochemical analysis provided insight into the MHC isoform composition of single Dia m fibers at D0 (Fig. 2). In the neonatal Dia m, MHCneo was the predominant isoform expressed, comprising ~67% of total MHC expression and being expressed in ~92% of all fibers. Expression of MHCslow constituted ~18% of total MHC, and singular expression was noted in only ~8% of all fibers at D0. Expression of MHC2A accounted for ~15% of total MHC, and there were no fibers that singularly expressed MHC2A at D0. Even by D14, no Dia m fibers were found that singularly expressed MHC2A. Expression of MHC2X was not detected until D14, and singular expression of MHC2X was not observed by single fiber SDS-PAGE. Overall, it appeared that most Dia m fibers continued to coexpress MHC isoforms at D14, with the exception of ~10% of all fibers that singularly expressed MHCslow. On the basis of single-fiber SDS-PAGE, the relative MHC isoform composition of single Dia m fibers was evaluated, and the relative coexpression of MHC isoforms was found to vary at D14. There was a predominant expression of one isoform (e.g., >40% of total expression of different MHC isoforms within single fibers could be determined with these methods.

Statistical analysis. A two-way ANOVA was used to make comparisons 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 across developmental ages. At D28 and in the adult rat Dia m, where singular MHC isoform expression occurs, comparisons were made across fibers expressing different MHC isoforms. At D14, coexpression of MHCslow, MHC2A, MHC2X, and MHCneo occurs along with singular expression of MHCslow. At this developmental time point, single fibers were grouped according to the dominant MHC isoform expressed. The relative proportion of MHC isoforms within single fibers at D14 was reported (Table 1). At D0, the majority of fibers expressed the MHCneo isoform (~90% according to immunohistochemical data); therefore, these fibers were considered relatively uniform, and a comparison across fibers within this developmental time point was not made. When appropriate, a Student’s t-test with Bonferroni correction was used for post hoc analyses. Values are reported as means ± SE. Statistical significance was indicated by a P value of <0.05.

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Fig. 1. Myosin heavy chain (MHC) isoform expression within rat diaphragm muscle (Dia m) single fibers was determined by SDS-PAGE. Day 14 (D14) single fibers are shown in lanes 1 and 2 and adult single fibers in lanes 3–6. Neo, neonatal.
MHC), and this formed the basis for categorization of Dia\textsubscript{m} fibers at D14 (Table 1). Fibers predominantly expressing MHC\textsubscript{2A} exhibited a variety of coexpression patterns as described in Table 1.

By D28, MHC\textsubscript{neo} expression was no longer present in the rat Dia\textsubscript{m}, and singular expression of MHC\textsubscript{slow}, MHC\textsubscript{2A}, and MHC\textsubscript{2X} isoforms occurs. Of the fibers sampled in the present study, 34\% expressed MHC\textsubscript{slow}, 46\% expressed MHC\textsubscript{2A}, and 20\% expressed the MHC\textsubscript{2X} isoform. This reflects the sample of fibers dissected and does not represent the overall proportion of MHC isoforms in the Dia\textsubscript{m}. As previously described in the adult rat Dia\textsubscript{m} (10, 37), singular expression of MHC\textsubscript{slow}, MHC\textsubscript{2A}, and MHC\textsubscript{2X} isoforms occurs in \~70\% of single fibers with coexpression of MHC\textsubscript{2X} and MHC\textsubscript{2B} isoforms in \~30\% of single fibers. For comparison with developmental isoform expression, adult Dia\textsubscript{m} fibers expressing the MHC\textsubscript{2X} isoform alone or coexpressed with the MHC\textsubscript{2B} isoform were classified as MHC\textsubscript{2X}. Singular expression of the MHC\textsubscript{2B} isoform was not detected in this study. Of the adult Dia\textsubscript{m} fibers sampled in the present study, 32\% expressed MHC\textsubscript{slow}, 15\% expressed MHC\textsubscript{2A}, and 53\% expressed MHC\textsubscript{2X}.

Cross-sectional area. Accurate cross-sectional area measurements were important for the measurement of MHC content per half-sarcomere and \(F_{\text{max}}\). Fiber cross-sectional area was therefore measured while the fiber was mounted on the stage of an inverted microscope at a set sarcomere length of 2.5 \(\mu\text{m}\). The \(xy\)-axis measurements were made using the \(\times40, 0.55\)-NA objective and were accurate within 0.5 \(\mu\text{m}\), whereas the \(z\)-axis distortion (~20\%) was corrected according to previous measurements made with confocal microscopy (9). In the present study, cross-sectional area measurements were made on 13 rat Dia\textsubscript{m} bundles and 246 single fibers. A dramatic increase in the cross-sectional area of Dia\textsubscript{m} fibers occurred during early postnatal development (Fig. 3A). The average cross-sectional area of D0 Dia\textsubscript{m} fibers was determined from images of D0 muscle cross sections used for immunohistochemistry. The cross-sectional area of individual D0 fibers was \~200
µm². The cross-sectional areas of fibers singularly expressing MHC slow at D0 were not significantly different from those of fibers coexpressing MHC slow together with MHC neo and MHC 2A. Fibers predominantly expressing MHC 2X at D14 had a slightly greater cross-sectional area than fibers predominantly expressing MHC 2A, MHC slow, or MHC neo, although the difference was not significant. At D28, the cross-sectional area of fibers singularly expressing the MHC 2X isoform was significantly greater than that of fibers singularly expressing MHC slow and MHC 2A isoforms. However, the difference in cross-sectional area between fibers expressing the MHC 2X isoform and other MHC isoforms at D28 was not as pronounced as that seen in the adult rat Dia m (Fig. 3A).

**MHC content per half-sarcomere.** MHC content per half-sarcomere was determined in 16 rat Dia m bundles and 127 single fibers. During early postnatal development, MHC content per half-sarcomere did not change, with the exception of fibers predominantly expressing MHC 2X (Fig. 3B). However, from D28 to adulthood, there was a dramatic increase in MHC content per half-sarcomere across all fibers. This increase in MHC content per half-sarcomere between D28 and adulthood was most pronounced in fibers expressing MHC 2X.

In Dia m bundles at D0, MHC content per half-sarcomere was comparable with that found in single fibers at D14, regardless of MHC isoform expression. However, even at D14, fibers predominantly expressing MHC 2X tended to have greater MHC content compared with other fibers. If corrected for the fact that MHC 2X expression accounted for only ~50% of total MHC expression at D14 (Table 1), it is likely that MHC 2X content per half-sarcomere was higher even at this early age. This is supported by the fact that the MHC content per half-sarcomere of fibers singularly expressing MHC 2X at D28 was significantly higher than that found for D14 fibers. This is in contrast to the switch from coexpression to singular expression of MHC 2A and MHC slow between D14 and D28, when MHC content per half-sarcomere remained constant.

F max was measured in 13 rat Dia m bundles and 246 single fibers and found to increase dramatically with postnatal development (Fig. 4). F max of Dia m bundles at D0 was significantly lower than that at all other developmental ages. The F max of fibers predominantly expressing the MHC 2X at D14 was significantly greater than that of fibers predominantly expressing MHC 2A and MHC neo as well as that of fibers singularly expressing MHC slow. These differences in F max were even more pronounced at D28, when fibers singularly expressed MHC isoforms. At D28, fibers expressing MHC 2X generated the greatest F max followed in rank order by fibers expressing MHC 2A and MHC slow. However, it should be noted that F max did not significantly increase between D14 and D28 for any fiber type. In contrast, between D28 and adulthood, F max increased by ~30% across all fibers, regardless of MHC isoform expression. Continuing the pattern first noted at D14, fibers expressing MHC 2X generated the greatest F max in the adult Dia m compared with fibers expressing MHC slow and MHC 2A isoforms.

**Force per half-sarcomere MHC content.** Maximum force values of rat Dia m bundles and single fibers were normalized for MHC content per half-sarcomere to determine the effect of cross-bridge number on F max. With the exception of fibers predominantly expressing MHC 2X, there was no change in force per half-sarcomere MHC content between D0 and D14 (Fig. 5). At D14, the force per half-sarcomere MHC content of fibers predominantly expressing MHC 2X was significantly greater than that of fibers predominantly expressing MHC 2A and MHC neo, as well as that of fibers singularly expressing MHC slow. Between D14 and D28, the force per half-sarcomere MHC content of fibers expressing MHC slow did not change. In contrast, the force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly. The force per half-sarcomere MHC content of fibers expressing MHC 2A increased significantly.
0.04. The average fraction of cross bridges in the force-generating state in D28 and adult fibers was 0.75 ± 0.02 and 0.75 ± 0.02, respectively. All values represent means ± SE. No significant differences in the fraction of cross bridges in the force-generating state were found across fibers expressing different MHC isoforms or across developmental time points in the rat Dia_m.

**DISCUSSION**

The results of the present study provide new information concerning the progressive increase in F_max during early postnatal development at the level of single muscle fibers. Between D0 and D28 in the rat Dia_m, differences in single fiber F_max emerge, such that fibers expressing fast MHC isoforms generate greater F_max compared with fibers expressing MHC slow. These fiber-type differences in F_max cannot be attributed to differences in MHC content per half-sarcomere. The results of the present study suggest that force per cross bridge is higher for fibers expressing fast MHC isoforms.

**MHC isoform expression.** A decrease in MHC_neo expression and a corresponding increase in fast MHC isoform expression characterize early postnatal development in the rat Dia_m. Furthermore, coexpression of MHC_neo with adult MHC isoforms is characteristic of Dia_m fibers through the first 2 postnatal wk. At D14, singular expression of MHC isoforms was limited to MHC_slow; however, by D28, most Dia_m fibers singularly expressed MHC isoforms. These results are in general agreement with previous observations (18–20, 35, 36, 44–47). However, the present study provides important new information regarding the relative coexpression of MHC isoforms in single fibers during postnatal development. Such information can only be obtained by single-fiber analysis. Unfortunately, at D0, it was not possible to reliably dissect the extremely small (~200 μm²) and fragile fibers in the rat Dia_m. However, the predominant expression of MHC_neo at this time point agrees with previous results from our laboratory (18, 35, 36, 44–47). At D14, although there was typically a predominant MHC isoform expressed (i.e., ~40% of total MHC), no fibers were found that coexpressed MHC isoforms in only trace amounts (i.e., <10%). Indeed, it was common for three MHC isoforms (e.g., MHC_neo, MHC_slow, and MHC_2A) to be coexpressed in relatively equal amounts. It remains unresolved whether MHC isoforms are coexpressed equally along the length of fibers or whether local regions [e.g., a “nuclear domain” (13, 25)] comprise predominantly a single isoform.

**Fiber cross-sectional area.** At D0 and D14, the cross-sectional areas of Dia_m fibers were relatively homogeneous (<10% coefficient of variation). By D28, the cross-sectional area of Dia_m fibers expressing the MHC_2X isoform was ~30% greater than that of fibers expressing MHC_2A and MHC_slow isoforms. In adults, Dia_m fibers expressing MHC_2X were three times larger than fibers expressing MHC_2A and MHC slow. These results are consistent with previous reports of postnatal growth of Dia_m fibers (46, 47).

Coexpression of MHC isoforms during postnatal development may confound fiber-type differences in cross-sectional area. At D14, Dia_m fibers predominantly expressing MHC_2X were slightly larger than fibers predominantly expressing other MHC isoforms. This difference was not significant, however, perhaps due to the extent of MHC_neo and MHC_2A coexpression in these fibers (~50% of total MHC; Table 1). As shown in Fig. 3A, changes in Dia_m fiber cross-sectional area were relatively minor until D28, when significant hypertrophy of fibers singularly expressing MHC_2X occurred. There was no significant growth of fibers expressing MHC_2A and MHC slow isoforms between D14 and D28. Most impressive was the dramatic but disproportionate growth of Dia_m fibers from D28 to adulthood (D84). However, this increase in fiber cross-sectional area in the rat Dia_m from D28 to adulthood cannot be explained by transitions in MHC isoforms, since such transitions were already complete by D28.

**MHC content per half-sarcomere.** MHC content per half-sarcomere in rat Dia_m fibers increased with postnatal development, with the greatest increase in fibers expressing the MHC_2X isoform. At D14, there was no significant difference in MHC content per half-sarcomere across fibers expressing different MHC isoforms. However, the substantial coexpression of MHC_neo and MHC_2A in D14 fibers predominantly expressing MHC_2X may have diluted any isoform-dependent difference in MHC content per half-sarcomere in these fibers. By D28, MHC content per half-sarcomere in fibers singularly expressing MHC_2X was ~50% greater than that in fibers singularly expressing MHC_2A and MHC slow isoforms. It is important to note that MHC content per half-sarcomere in fibers expressing MHC_2A and MHC slow did not increase significantly until adult-
hood when a significant increase in MHC content per half-sarcomere occurred across all fiber types. Like the dramatic increase in cross-sectional area between D28 and adulthood, this increase in MHC content per half-sarcomere across all fiber types cannot be attributed to transitions in MHC isoform expression.

As previously shown (9), these results provide further support for a lower MHC content per half-sarcomere in fibers expressing MHCA2A and MCHslow compared with fibers expressing MHCA2X. These findings are consistent with a higher mitochondrial volume density in fibers expressing MHCA2A and MCHslow isoforms compared with fibers expressing MHCA2X and MHCA2B isoforms (33).

Fmax increased during postnatal development in the rat DiaM across all fiber types. These results are consistent with the increase in Fmax of DiaM fiber bundles reported in previous studies (18, 35, 46, 47). Obviously, these results at the single-fiber level are an important extension of these previous observations. Of particular interest, Fmax increased dramatically from D0 to D14 across all MHC isoforms, whereas fiber cross-sectional area and MHC content per half-sarcomere remained constant. Thus fiber-type differences in Fmax precede fiber-type differences in cross-sectional area and MHC content per half-sarcomere in the developing rat DiaM.

The results indicating fiber-type differences in specific force in the rat DiaM, even at D14, are consistent with previous reports from this laboratory (9, 33, 34) as well as other studies in the rat DiaM (6) and in limb muscles (1, 22). However, it should be pointed out that some controversy does exist as to whether fiber-type differences in specific force exist across all muscles (8). A number of studies examining fiber-type differences in specific force have compared fibers from a predominantly fast-twitch muscle with fibers from a predominantly slow-twitch muscle. Differences in physiological function, activation history, and load can influence the contractile properties of these muscles. Few studies have examined fiber-type differences in specific force within the same muscle to eliminate such confounding factors.

Previous studies from our laboratory indicated a strong correlation between postnatal changes in Fmax of rat DiaM fiber bundles and the relative expression of fast MHC isoforms (18, 35). However, these studies also warned that factors other than changes in the relative expression of fast MHC isoforms must also influence the postnatal changes in Fmax. For example, Johnson and colleagues (18) found very little change in the relative expression of fast MHC isoforms between D3 and D7 and between D21 and adulthood, whereas Fmax of DiaM fiber bundles displayed the greatest increase during these time periods. Similarly, MHC isoform expression did not change significantly between D28 and adulthood in the present study; however, single-fiber Fmax increased dramatically during this time period. In fact, the greatest transition in MHC isoform expression in DiaM fibers occurs between D14 and D28, yet Fmax of single fibers does not significantly increase during this time period. Thus the increase in Fmax during postnatal development of rat DiaM fibers does not appear to be solely dependent on MHC isoform expression.

**Force per MHC content.** In the present study, normalizing maximum DiaM fiber force for half-sarcomere MHC content did not eliminate differences in force associated with MHC isoform expression or postnatal development. At D28 and in adults, force per half-sarcomere MHC content was greater in fibers expressing MHCA2A and MCHslow isoforms compared with fibers expressing MCHslow. These results are consistent with our previous observations in the adult rat DiaM (9). These results indicate that fibers expressing MHCA2A and MCH2X isoforms have greater force per cross bridge than fibers expressing MCHneo and MCHslow isoforms.

Between D0 and D14, force per half-sarcomere MHC content increased by ~50%, but only in DiaM fibers predominantly expressing MHCA2X. Of interest is the fact that, at D14, the force per half-sarcomere MHC content was not greater in DiaM fibers predominantly expressing the MHCA2A isoform. However, between D14 and D28, the force per half-sarcomere MHC content in DiaM fibers predominantly expressing the MHCA2A isoform increased dramatically, approximating that of fibers expressing MHCA2X. The underlying basis for this difference in the development of force-generating capacity in fibers expressing fast MHC isoforms remains unresolved.

It is clear from the results of the present study that factors other than MHC isoform expression contribute to both fiber-type and postnatal differences in Fmax. One possibility is that the lateral spacing of the filament lattice may vary with fiber-type and postnatal development and thus affect the probability of cross-bridge attachment necessary for force generation. It is also possible that there are developmental transitions in the expression of structural proteins important in maintaining sarcomere stability and thus force generation. For example, titin is a large structural protein in skeletal muscle fibers that provides a major contribution to passive mechanical properties (14, 42) as well as positional stability of thick filaments during isometric contraction (15). It has been demonstrated that different isoforms of titin exist in fast-twitch vs. slow-twitch skeletal muscles, and these isoforms differ in their elasticity (41). Differences in titin isoform expression in rat DiaM fibers have not been explored. It is also unknown whether there are postnatal transitions in titin isoform expression. It is possible that changes in titin isoform expression could contribute to the differences in Fmax across DiaM fibers and the changes in Fmax during postnatal development.**

**Influence of cross-bridge cycling kinetics on specific force.** On the basis of Huxley’s two-state model of cross-bridge cycling (17), cross bridges cycle between 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. It might be expected that, with slower cross-bridge cy-
clinging kinetics and a longer duty cycle for cross-bridge attachment, there would be an increase in the fraction of strongly bound cross bridges and a greater $F_{\text{max}}$ (12). To evaluate this possibility in the present study, the fraction of strongly bound cross bridges was estimated from measurements of muscle fiber stiffness. However, the fraction of strongly bound cross bridges during maximum $Ca^{2+}$ activation did not change during early postnatal development, and thus this could not account for the lower $F_{\text{max}}$ generated by fibers expressing MHC$_{\text{slow}}$ and MHC$_{\text{neo}}$. These results are consistent with our laboratory’s previous studies in the adult rat Dia$_{\text{m}}$, in which no difference in the fraction of strongly bound cross bridges during maximum $Ca^{2+}$ activation was found across fibers expressing different MHC isoforms (9, 34) despite an approximately twofold difference in cross-bridge cycling kinetics (as estimated by the rate of force redevelopment after rapid release and restretch) (34). In a previous study on the superfasciculated swim bladder muscle in toadfish, Rome and colleagues (26) reported that the superfasciculated cross-bridge cycling kinetics of this muscle was associated with a lower fraction of strongly bound cross bridges during maximum activation and reduced specific force. However, these investigators also reported that white (fast-twitch) muscle fibers in the toadfish actually generated slightly greater specific force than red (slow-twitch) muscle fibers despite an approximately threefold difference in cross-bridge cycling kinetics.

Force per cross bridge has been estimated from in vitro motility assays measuring unitary force. The results of previous studies examining unitary force in cardiac V1 and V3 myosins have been controversial, with some studies reporting no difference in force (5, 28, 38) and others reporting that V3 myosin generates greater force than V1 (21, 27). These discrepant results could be due to incorrect orientation of myosin molecules in the in vitro system or differences in the actual number of myosin molecules involved in the force measurement. These are all problems inherent to the in vitro assay, and further work is needed to determine potential differences in unitary force between myosin molecules.

Functional significance. Neonatal rats demonstrate increased respiratory rates and lower tidal volumes compared with adults (24). The reliance on rapid, shallow breaths relates in part to the distortion of the highly compliant neonatal chest wall. It may also reflect the inability of the neonatal Dia$_{\text{m}}$ to generate sufficient force to accommodate deeper breaths. During development, the increase in Dia$_{\text{m}}$ force-generating capacity coincides with the development of a more stable chest wall. The Dia$_{\text{m}}$ becomes more heterogeneous after the emergence of MHC$_{2X}$ isoform expression, and this coincides with the capacity for differential recruitment of motor units (31). In the adult Dia$_{\text{m}}$, quiet breathing is accomplished by the recruitment of fatigue-resistant motor units comprising fibers that express MHC$_{\text{slow}}$ and MHC$_{2A}$ isoforms (29, 30, 32). Only during short-duration expulsive motor behaviors of the Dia$_{\text{m}}$ (e.g., coughing) is it necessary to recruit the more fatigable fast motor units comprising fibers that express MHC$_{2X}$ and MHC$_{2B}$ isoforms. During early postnatal development (up to D14), polyneuronal innervation of Dia$_{\text{m}}$ fibers exists (31), and this limits the efficacy of differential motor unit recruitment. It is likely that the coincidence of developmental changes in innervation pattern, MHC isoform expression, and force-generating capacity in Dia$_{\text{m}}$ fibers supports the emergence of a wider range of functional requirements and motor behaviors characteristic of the adult.

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REFERENCES


6. Eddinger TJ and Moss RL. Single-gene differences in myosin molecules in the in vitro system or differences in the actual number of myosin molecules involved in the force measurement. These are all problems inherent to the in vitro assay, and further work is needed to determine potential differences in unitary force between myosin molecules.


