Diaphragmatic Muscle Fiber Type Development in Swine

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ABSTRACT. Diaphragmatic muscle fiber types were determined in the costal and crural segments of swine diaphragm at 4 postnatal ages (1 day, 1 month, 6 months, and between 3–6 yr of age). Fiber types were differentiated by enzyme histochemistry for adenosine triphosphatase and reduced nicotinamide adenine dinucleotide. A progressive increase in the number of type I fibers occurred in both costal and crural segments from birth to 6 months of age. The number of type IIA fibers decreased and type IIB fibers increased over the same time period. Type IIC fibers were present through 1 month of age, were rarely observed at 6 months, and were not found in older animals. Type I fibers were more numerous in the crural portion of the diaphragm. The cross-sectional area of all fiber types in both costal and crural segments increased significantly with age. No preferential fiber type growth was noted in either segment of the diaphragm. These data suggest that the pig diaphragmatic muscle is differentiated into its adult form by 6 months of postnatal age, but fiber cross-sectional area growth continues along with body growth. (*Pediatr Res* 22: 449–454, 1987)

Abbreviations

ATPase, adenosine triphosphatase
NADH-TR, reduced nicotinamide adenine dinucleotide tetrazolium reductase

Respiratory failure is a common problem in the neonate and one that accounts for significant mortality and morbidity during this early stage in life. The pathophysiologic mechanisms that lead to respiratory failure and the need for ventilatory support are not clearly defined. The major muscle of respiration, the diaphragm, has been the focus of intensive investigation recently in an attempt to define these mechanisms. However, most studies have utilized adult animals and humans to elucidate the causes of respiratory failure (1–8). Little attention has been focused on the neonate. In adult humans and animals, the response of certain muscle groups to various stresses can be related to their fiber type composition (2, 9–11).

The newborn piglet has been used extensively in our laboratory as a model in which to study diaphragmatic muscle function in a developmental context (12–17). The normal pattern of peripheral skeletal muscle development in the pig that occurs with postnatal maturation has been described (18–23). However, the

pattern of respiratory muscle fiber type development has not been delineated. We examined the fiber type distribution and cross-sectional area in the swine diaphragm during postnatal development from the neonate to the adult in an effort to provide anatomic correlates for physiologic studies of diaphragmatic function in the developing piglet.

MATERIALS AND METHODS

Tissue sampling. Specimens of the left costal and crural diaphragm from four Yorkshire pigs were obtained at four postnatal ages. Two male and two female animals were studied at 1 day, 1 month, and 6 months of age. Four sows were studied at 3–6 yr of age. Samples were taken immediately following sacrifice with intraperitoneal pentobarbital overdose. The diaphragm was rapidly exposed and muscle was sampled from the left anteriolateral portion of the costal diaphragm midway between the central tendon and the rib insertion. Samples of the left crural diaphragm were obtained from the midportion of the muscle belly. The sample sizes were approximately $1 \times 3 \times 3$ mm. The muscle samples were mounted on cork at approximately resting length and quick frozen in isopentane cooled in liquid nitrogen. They remained frozen at minus 70° C until sectioned.

Histochemistry. Frozen specimens were brought to a temperature of minus 20° C and serial 10- μ m cross-sections were cut on an American Optical cryostat (American Optical Corporation, Buffalo, NY). Sections were dried at room temperature for 30–60 min and then incubated in a battery of histochemical media. Samples of human peripheral skeletal muscle obtained from infants and adults for other studies were incubated simultaneously and served as controls for the histochemical reactions. Muscle samples from the day-1, 1-month, and 6-month-old swine were batched for staining. All samples that visually demonstrated unacceptable sectioning or histochemical staining were discarded and repeated. Additionally, if unacceptable staining was found in the internal controls, the swine samples were discarded and repeated. Samples from the adult swine were incubated in the histochemical media simultaneously with control adult human muscle samples. Reactions were performed to demonstrate the activities of NADH-TR and myofibrillar ATPase. The NADH-TR assay was performed according to the method of Dubowitz and Brooke (24). Myofibrillar ATPase assay was performed as described by Chayen *et al.* (25). Serial sections were preincubated at alkaline pH (10.3) and at acid pH (4.5 and 4.3) prior to the myofibrillar ATPase assay (24). The pH of the incubation solutions was adjusted at 37° C.

Fibers were visually typed according to the schema in Figure 1. Initially, the fibers were classified as either type I or II by their ATPase staining intensity following preincubation at a pH of 10.3. Type I fibers demonstrate no reaction at this pH while type II fibers stain intensely. Type II fibers were then subgrouped by their ATPase differential staining reaction following preincuba-

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tion at pH 4.3 and 4.5. It was not possible to subgroup type II fibers utilizing the NADH-TR assay as all fibers stained intensely by this method on day 1 of life as previously documented (18–21, 23) (see Fig. 2). Similar staining occurred in most specimens at 1 month of age (five of eight costal specimens). Therefore, serial sections were compared for differential staining by the ATPase assay. The NADH-TR and ATPase assays were utilized for differential fiber typing in the specimens of diaphragm obtained at 6 months and 3–6 yr. Drawings of microscopic projections of the muscle sections were made utilizing a Zeiss micro-

scope and drawing tube (Carl Zeiss, Inc., Thornwood, NY) and identical fields from serial sections of the muscle specimen were overlaid on the drawing to allow for subgrouping of the fiber types. Between 127 and 742 (mean = 453) fibers were traced per tissue sample.

Cross-sectional area. The cross-sectional area of each fiber was determined utilizing a Numonics digitizing tablet and electronic graphics calculator model 2400 (Numonics Corporation, Lansdale, PA). Fiber outlines were traced on the drawings previously

Table 1. *Diaphragm muscle fiber type composition**

	Type I	Type IIA	Type IIB	Type IIC
	%	%	%	%
Costal				
Day 1	18 ± 2	61 ± 5	10 ± 2	11 ± 2
1 mo	29 ± 3	56 ± 4	3 ± 1	13 ± 3
6 mo	49 ± 4	31 ± 4	20 ± 7	1 ± 1
3–6 yr	48 ± 3	24 ± 6	28 ± 4	0
Crural				
Day 1	20 ± 1	61 ± 2	9 ± 1	10 ± 2
1 mo	39 ± 2	43 ± 3	3 ± 1	15 ± 1
6 mo	55 ± 3	30 ± 5	13 ± 4	2 ± 1
3–6 yr	58 ± 5	20 ± 4	23 ± 7	0

* $n = 4$ for each group. Percentage of each fiber type given as the mean $\pm$ SEM. The total percent of fibers may exceed 100% due to rounding error. See text for further details.

	Muscle Fiber Type			
	1	2 A	2 B	2 C
ATP-ase Preincubated pH 10.3
ATP-ase Preincubated pH 4.5
ATP-ase Preincubated pH 4.3
NADH - TR

Fig. 1. Histochemical reaction intensities in swine diaphragm. Scheme for classification of muscle fibers according to relative reaction intensity. See "Materials and Methods" for further details.

Fig. 2. Muscle fiber histochemical reactions on day 1 in swine. Representative costal diaphragm muscle sample on day 1 of life. NADH-TR and ATPase histochemical reactions. Original magnification is $\times 64$. A, NADH-TR: all fibers stain intensity precluding fiber subtyping. B, ATPase at pH 10.3: Dark fibers are type II, light staining fibers are type I. C, ATPase at pH 4.5: representative fibers are labeled. D, ATPase at pH 4.3: representative fibers are labeled.

made from the ATPase reaction slide preincubated at pH 10.3 and the area determined by the Numonics calculator was converted to square microns utilizing a micrometer slide as a standard at a specific magnification. The area of 50 fibers in each fiber type subgroup in each sample were determined if possible.

Results were analyzed by analysis of variance for fiber type or cross-sectional area versus age and location utilizing Minitab (Release 82.1, 1984, PC version).

RESULTS

Table 1 lists the fiber type composition of the two segments of the diaphragm at the four study ages. Representative sections from day-1 (Fig. 2), 1-month-old (Fig. 3), and 6-month and 3- to 6-yr old animals (Fig. 4) are included. No significant differences were noted between the costal and crural segments for type II fibers at any specific age. A significant progressive increase in type I fibers in both segments of the diaphragm was noted until 6 months of age ($p < 0.001$). No further increase was noted in the specimens from animals at 3–6 yr of age. Type I fibers were more numerous in the crural compared to the costal diaphragm ($p < 0.02$). The quantity of type IIA fibers progressively decreased in both segments of the diaphragm through 6 months ($p < 0.001$). Type IIB fiber quantity averaged 10% at birth, appeared to decrease slightly at 1 month of age, then increased in both segments of the diaphragm to 3–6 yr ($p < 0.001$). A small proportion of type II fibers did not demonstrate acid reversal and were classified as type IIC. The percent of type IIC fibers decreased as the animals matured, were rarely seen at 6 months, and were not observed at 3–6 yr of age.

All fibers significantly increased in cross-sectional area with postnatal maturation (Fig. 4; Tables 2 and 3). No differences in area were found between the costal and crural segments of the diaphragm for any fiber type subgroup at each age. On day 1 of life, the fibers were round in shape (Fig. 2). The profile of type I fibers remained round with age whereas type II fibers became larger and more polygonal with maturation (Fig. 4).

DISCUSSION

The normal postnatal changes in fiber types and cross-sectional area of the costal and crural diaphragmatic muscle in the piglet have been determined. Type I fiber quantity was found to increase with maturation to 6 months of age. Type IIA fiber quantity decreased through 6 months of age while type IIB fiber quantity increased. Type IIC fibers comprised approximately 10% of all fibers at birth, were rarely seen by 6 months, and were not found in the adult pig. All fibers demonstrated a significant increase in cross-sectional area that continued to the 3- to 6-yr group of animals.

Critique. Fiber type determination by histochemistry is a standardized methodology when used to examine specimens of mature muscle (2–4, 7, 8, 10, 24). However, questions have been raised in regards to the applicability of this methodology to developing muscle in the fetus and newborn (26). Guth and Samaha (26) demonstrated a discrepancy between ATPase histochemical staining of newborn rabbit muscle and the biochemical activity of the ATPase enzyme. Additionally, Guth (27) found that the histochemical demonstration of myofibrillar ATPase activity could be misleading since the reaction product may

Fig. 3. Muscle fiber histochemical reactions at 1 month in swine. Representative costal diaphragm muscle sample at 1 month of life. NADH-TR and ATPase histochemical reactions. Original magnification is $\times 64$. A, NADH-TR: differential staining fibers is apparent. However subgrouping cannot be readily done. B, ATPase at pH 10.3: dark fibers are type II, light staining fibers are type I. C, ATPase at pH 4.5: representative fibers are labeled. D, ATPase at pH 4.3: representative fibers are labeled.

Fig. 4. Fiber type cross-sectional area development in swine. Representative costal diaphragm muscle samples at the four different ages studied. ATPase histochemical reactions following preincubation at pH 10.3. Original magnification is $\times 64$. A, day 1; B, 1 month; C, 6 months; D, 4 yr.

Table 2. Fiber cross-sectional area*

Costal diaphragm								
	Type I	<i>n</i>	Type IIA	<i>n</i>	Type IIB	<i>n</i>	Type IIC	<i>n</i>
Day 1	138 $\pm$ 14	59	204 $\pm$ 57	61	150 $\pm$ 30	49	139 $\pm$ 20	55
1 mo	656 $\pm$ 109	50	733 $\pm$ 108	50	441 $\pm$ 62	13	453 $\pm$ 78	41
6 mo	4387 $\pm$ 464	50	4275 $\pm$ 451	50	5413 $\pm$ 478	41	4211 $\pm$ 1076	4
3-6 yr	6862 $\pm$ 539	50	5038 $\pm$ 396	42	5962 $\pm$ 1170	50		0

* Area is given in μ^2 . The mean and SE of the cross-sectional area are given for each age group and fiber type. *n* is the mean number of fibers of a specific type counted per animal. All fiber types significantly increased in cross-sectional area (two-way analysis of variance, $p < 0.001$).

Table 3. Fiber cross-sectional area*

Crural diaphragm								
	Type I	<i>n</i>	Type IIA	<i>n</i>	Type IIB	<i>n</i>	Type IIC	<i>n</i>
Day 1	119 $\pm$ 13	50	145 $\pm$ 10	51	125 $\pm$ 7	47	102 $\pm$ 7	42
1 mo	568 $\pm$ 39	50	624 $\pm$ 35	50	452 $\pm$ 10	14	366 $\pm$ 28	50
6 mo	4268 $\pm$ 230	50	4236 $\pm$ 169	50	5157 $\pm$ 76	38	2897 $\pm$ 309	9
3-6 yr	6927 $\pm$ 860	50	5855 $\pm$ 911	48	6141 $\pm$ 1266	36		0

* Area is given in μ^2 . The mean and SE of the cross-sectional area are given for each age group and fiber type. *n* is the mean number of fibers of a specific type counted per animal. All fiber types significantly increased in cross-sectional area (two-way analysis of variance, $p < 0.001$).

not have originated solely in the myofibrils themselves, but also in mitochondria. However, more recent workers have found good correlations between myofibrillar ATPase staining and oxidative staining over a wide age range in baboon ventilatory muscles (28). Maxwell *et al.* (28) found that all fibers in the fetal baboon stained intensely for NADH-TR. Similar findings were reported in piglets (18-23) and in kittens (29). These later studies

relied on myofibrillar ATPase activity for fiber type classification rather than oxidative enzyme reactions. The histochemical studies in the baboon demonstrating high oxidative capacity of all fibers in the respiratory muscles was in excellent agreement with measures of function (28) and is the first study to correlate diaphragmatic muscle function with histochemical findings within a developmental context. We conclude that the histo-

chemical methodology utilized in this study accurately identifies muscle fiber types in the newborn piglet.

Suzuki and Cassens (30) found that the histochemical demonstration of ATPase enzymatic activity was very pH sensitive. These results lead us to utilize the preincubation of pHs of 10.3, 4.5, and 4.3 to carefully differentiate fiber type subgroups and allow for a second check to define type IIB fibers.

Maturation changes in muscle fiber types. Previous studies of postnatal changes in fiber types in the pig have focused primarily on peripheral skeletal muscle (18–23). The developmental changes in muscle fiber type distribution in the pig, both in peripheral skeletal muscle and in the costal diaphragm have been described (18). As in the present study and several previous investigations (18, 19, 23), all fibers demonstrated high oxidative histochemical activity, as evidenced by NADH-TR enzyme staining, for several weeks after birth. The postnatal developmental changes in fiber type distribution were similar to the present study, but cannot be compared directly to the present study since the age of the piglets studied were not given, tissue preparation was different, and the histochemical techniques were not identical. Suzuki and Cassens (22) studied myofiber type development from birth to 16 wk of age in swine peripheral skeletal muscle. They found a progressive increase in type I fiber quantity with a decrease in type II fibers out to 8 wk of life. However, the quantity of type I fibers in the longissimus, rectus femoris, and masseter muscles was very low compared to diaphragmatic muscle examined in this study. Fiber type distribution found in the diaphragm of our piglets more closely resembled those found in the trapezius muscle examined (22).

In the adult pig, Davies and Gunn (31) examined diaphragmatic muscle fiber type composition and found 45% type I, 20% type IIA, and 33% type IIB fibers, values in close agreement with our data. Similar type I fiber quantities in adult diaphragmatic muscle have been found in the cat (4, 7, 8) and the rat (3).

Maxwell *et al.* (28) examined the histochemical properties of baboon respiratory muscles from midgestation to adulthood. In fetal and neonatal muscle, they found a high percentage of type IIC fibers which stained intensely for oxidative enzymes. They concluded that the respiratory muscles of the baboon during gestation and in the neonatal period are high in oxidative capacity. However, the baboon respiratory muscles are substantially more differentiated at birth than in the human. This contrasts with previous studies which suggested that human muscle fiber type differentiation is more advanced than in other species (32–36).

Keens *et al.* (37) studied the development of human respiratory muscles from 24 wk gestation to adulthood. They found a progressive increase in type I fiber number in the diaphragm from 10% in infants less than 37 wk gestation to 25% in full term infants and 55% in subjects more than 2 yr of age. Adult levels of type I fibers were reached by 8 months of postnatal age. A similar progression was noted in samples from internal and external intercostal muscles with the exception that the percent of type I fibers was greater at any age and no further differentiation was noted after 2 months of life.

Type I fiber differentiation in swine resembles that found in the human, but with a shifted time frame (37). The findings in the pig at birth are similar to the premature human <37 wk gestation; at 1 month, swine are similar to the full-term human infant; swine at 6 months and older are similar to adult humans. When compared to the baboon (28), swine demonstrate a similar pattern of maturational change. However, it would appear that baboon diaphragmatic muscle fiber type differentiation is slightly more advanced at birth than in swine. The newborn pig has a similar fiber type distribution as the baboon at approximately 140 days with the exception that baboon type IIB fibers are not seen until after term. At 3–6 yr, swine have a similar quantity of type I, IIA, and IIC fibers, but less IIB fibers when compared to the baboon.

Maturation changes in fiber cross-sectional area. The values

reported by Davies and Gunn (31) for fiber cross-sectional area as determined by planimetry at 10 and 56 days postnatal age would fit with our data for the costal diaphragm. The mean fiber area in their six pigs was $5680 \pm 1170 \mu^2$, very similar to those found in the present study. This fiber size was significantly larger than values found in the horse, ox, sheep, dog, cat, rabbit, rat, or mouse. The large area of "myosin-ATPase low" or type I fibers in the costal diaphragm of their pigs and that found in the present study contrasts with previous work that suggested that type I fibers in peripheral skeletal muscle were generally of small cross-sectional area probably to allow for adequate substrate delivery (8). Sieck, *et al.* (8) determined that the type II fibers of the adult cat diaphragm were generally larger than type I fibers, but substantial variability was reported in the cross sectional area of both fiber types.

Bowden and Goyer (38) studied fiber size developmental changes in human diaphragm muscle up to 9 yr of age. They found that diaphragm muscle fibers were larger than other striated skeletal muscle fibers until 9 yr of age. The samples that were obtained at autopsy in preterm and term infants have comparable fiber cross-sectional area to 1-day-old piglets in the present study. However, the piglet fibers were much larger at all succeeding ages as compared to the human.

The results of this study and that of Davies and Gunn (31) would suggest that each diaphragmatic muscle fiber can obtain sufficient substrate to function on an intermittent basis indefinitely despite their large cross-sectional area in the adult.

Relationship of fiber type to function. Hammarberg and Kellner (39) found that the hindlimb muscles of kittens were quite fatigue resistant and the adult pattern of fatigability only became evident by 40 to 70 days of postnatal age. This change over in fatigue pattern corresponded very closely to histochemical fiber type maturation (40) and loss of polyneuronal innervation (41, 42). Keens *et al.* (37) concluded that the human neonate is more susceptible to respiratory muscle fatigue based on the large percent of type II fibers present in the respiratory muscles. However, no functional measures were obtained. Maxwell *et al.* (28) examined the functional properties of baboon respiratory muscles from midgestation to adulthood. Functionally, the diaphragmatic muscle of the premature baboons was found to be more fatigue resistant than that of the adult. They concluded that the respiratory muscles of the baboon during gestation and in the neonatal period are high in oxidative capacity and are fatigue resistant. The finding of moderate to high oxidative activity in the neonatal human diaphragm by the same investigators provides further support for this argument (43).

Lieberman *et al.* (2) examined the adult guinea pig and human diaphragm. They found an excellent correlation of histochemical fiber type quantities with functional properties in both species. Additionally, they tested human adult males during maximal voluntary ventilation trials and concluded that the initial small decrease in ventilation noted within the first min of the trial was secondary to fatigue of low oxidative fast twitch fibers (or type IIB). The plateau in the maximal voluntary ventilation trial was maintained out to 5 min and correlated well with the total percent of high oxidative fibers, specifically type I and IIA fibers.

Biochemical measurement of oxidative enzyme activity is necessary for proper interpretation of work capacity of a muscle fiber under specified conditions. Therefore, we can only speculate as to the functional correlates of fiber type subgroups and cross-sectional area determined in this study. All fibers demonstrated intense oxidative enzyme histochemical staining early in life (Figs. 2 and 3). If one would consider the sum total of type I and type IIA fibers as being oxidative in nature, then this sum remains high (mean $80 \pm 4\%$) throughout the range of ages studied herein. These findings would suggest that the diaphragm should be relatively fatigue resistant throughout life in the pig. This hypothesis awaits further testing since the diaphragm has not been studied from a functional point of view in the adult pig. However, recent studies in the neonatal piglet have examined

the functional capabilities of the diaphragm in a developmental context. The force output of the diaphragm increases with postnatal age, body weight, and diaphragmatic wet weight for a given central drive to the muscle (14). Additionally, the neonatal piglet diaphragm can fatigue when confronted with large inspiratory resistive loads despite elevated central drive (12). A decrease in the force frequency curve of the diaphragm at 1 h of resistive loading occurred indicating fatigue despite the clinical observation that respiration continued, EMG significantly increased, and the spontaneously generated transdiaphragmatic pressure remained elevated. Thus, although fatigue can be uncovered by certain testing procedures, clinical manifestations may not be present.

In summary, the muscle fiber type subgrouping and cross-sectional area changes that occur in the costal and crural diaphragm with postnatal maturation in the piglet have been delineated. A progressive increase in the portion of type I fibers was found to 6 months of age. Type I fibers were more numerous in the crural segment compared to the costal segment of the diaphragm. Type IIA fibers decreased and type IIB increased over the same time period. Type IIC fibers represented approximately 10% of the total fiber quantity at birth, were rarely observed at 6 months of age, and were not found in the 3- to 6-yr old group of animals. Fiber type differentiation was, therefore, completed by 6 months postnatal age. All muscle fibers increased in cross-sectional area out to 3–6 yr of age. These results provide correlates for the study of respiratory muscle function in the piglet in a neonatal and developmental context.

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