0031-3998/92/3104-0354$03.00/0
PEDIATRIC RESEARCH
Copyright © 1992 International Pediatric Research Foundation, Inc.
Vol. 31, No. 4, 1992
Printed in U.S.A.

Glycogen Content in Neonatal Diaphragmatic Fibers in Response to Inspiratory Flow Resistive Loads[1]

YOUNG-JEE KIM AND ALIA R. BAZZY

Department of Pediatrics, Section of Respiratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT. To study intramuscular glycogen use with increased work loads in the neonatal diaphragm, unanesthetized lambs were subjected to severe inspiratory flow resistive loads. With these loads, transdiaphragmatic pressure and arterial CO_2 tension increased several fold above baseline and then remained stable for a period of 10–30 min. This was followed by a decrease in transdiaphragmatic pressure and a marked increase in arterial CO_2 tension with severe acidosis. Intramuscular glycogen content was determined histochemically by the periodic acid-Schiff reaction and was quantified microphotometrically by the fiber types (type I, IIa, or IIc) present in the lamb costal diaphragm. Glycogen content in the control diaphragm was greatest in type IIa fibers and lowest in type I fibers. With severe inspiratory flow resistive loads, the greatest decrease in glycogen content was in type IIa fibers, followed by type IIc and type I fibers. Therefore, our data suggest that in the neonatal lamb subjected to inspiratory flow resistive loads the diaphragm uses intramuscular glycogen during increased work loads. These data do not indicate that the marked depletion of glycogen in the diaphragm is the cause of the decrease in diaphragmatic force. (*Pediatr Res* 31: 354–358, 1992)

Abbreviations

IFR, inspiratory flow resistive
Pdi, transdiaphragmatic pressure
ROD, relative optical density
Pa_{CO_2}, arterial CO_2 tension
Pa_{O_2}, arterial O_2 tension
PAS, periodic acid-Schiff

It has been shown that the adult diaphragm uses intramuscular glycogen as an energy substrate during increased work of breathing (1–10) and that the magnitude of glycogen use depends on the severity of the work load (1, 3, 5, 8–10). It also depends on fiber type: glycogen depletion is greatest in type II fibers with severe work loads (1, 6, 10). Whereas glycogen use in the adult diaphragm has been relatively well explored, little is known about glycogen use in the newborn diaphragm. Because the proportion of type II fibers in the neonatal diaphragm is higher than in the adult diaphragm (11–14), dependency of the diaphragm on intramuscular glycogen may be greater in neonates than in adults. However, glycolytic enzyme activity in neonatal skeletal muscles (15–19) and diaphragm (20), as well as enzymes for glycogenolysis in neonatal skeletal muscles (17, 19, 21–25), has been shown to be low, whereas glycogen content in neonatal diaphragm and skeletal muscles has been demonstrated to be higher than (19, 26–29) or similar to that in adult muscles (16). These findings suggest that glycogen use in the neonatal diaphragm may be limited under conditions of increased energy demands. Therefore, the aim of our study was to examine whether intramuscular glycogen in the neonatal diaphragm is used during high energy requirements, focusing on the glycogen use pattern in individual diaphragmatic muscle fiber types.

MATERIALS AND METHODS

Six neonatal lambs, 4–14 d of age, were studied. All lambs were housed with their ewes in a facility maintained at 23–25°C on a 12:12-h light-dark cycle. Two d before each experiment, a tracheostomy was performed and a catheter was placed in the descending aorta through a femoral artery with the lamb under general anesthesia (halothane, N_2O, O_2). The tracheostomy was maintained with a No. 9 silicone rubber tracheal T-tube (E. Benson Hood Laboratories, Inc., Pembroke, MA), and the femoral artery catheter was flushed with saline and heparinized daily until the day of the experiment. Arterial blood gas tensions of tracheostomized lambs were measured daily to evaluate whether the tracheostomy affected ventilation. Arterial blood gas tensions of tracheostomized lambs were not different from those of nontracheostomized lambs. Also, there was no difference between respiratory rate before and after the tracheostomy. Body weight was closely monitored to ensure that the lambs were gaining postoperatively.

Physiologic measurement. On the day of the experiment, a cuffed endotracheal tube (Shiley No. 4, Shiley, Inc., Irvine, CA) was inserted via the tracheostomy site and connected to a nonrebreathing Hans Rudolph valve (Hans Rudolph, Inc., Kansas City, MO). Two thin-walled, 5 cm-long latex balloons were also placed in the stomach and in the middle third of the esophagus to measure gastric pressure (Pga) and esophageal pressure (Pes), respectively. These acute procedures were performed gently and quickly to reduce the stress induced by the intervention. About 30 min of recovery time was given after instrumentation, and recovery was confirmed by return of respiratory pattern and arterial blood gas tensions to preinstrumentation levels. Inspiratory flow rate was measured with a Fleisch No. 1 pneumotachograph (OEM Medical, Inc., Richmond, VA) and a differential air pressure transducer (PM15E; Statham, Cambridge, MA). A biased flow of 5 L/min of oxygen was manually set to zero because all lambs were supplemented with oxygen during exper-

Received August 20, 1990; accepted November 27, 1991.
Correspondence: Alia R. Bazzy, M.D., Department of Pediatrics, Section of Respiratory Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.
Supported by NIH Grant HL01518. Y.-J.K. is the recipient of American Heart Association, Connecticut Affiliate Fellowship Award, and A.R.B. is the recipient of an NIH Clinical Investigator Award.
[1] Presented in part at the annual meeting of the Society for Pediatric Research, Anaheim, CA, May 1990.

iments to prevent severe hypoxia and agitation. Pdi (Pdi = Pga − Pes) was measured by connecting the gastric and esophageal catheters to a differential air pressure transducer (Statham PM131TC). Pressure and airflow signals were continuously recorded on a polygraph (Gould TA 2000). Arterial blood gas tensions were also analyzed every 3 to 5 min during the course of the experiment from blood samples obtained from the indwelling aortic catheter. After obtaining baseline measurements, the lamb was exposed to an IFR load. The load, consisting of a narrow-bore plastic tube, was connected to the inspiratory limb of the valve, keeping the expiratory limb free. Each lamb underwent one study with one load. The resistance of the IFR load was calculated at the peak flow rate during the inspiratory cycle. The choice of the load was based on pilot experiments in which a resistance greater than 250 cm H_2O/L/s led to very little increase in Pdi from baseline, and a resistance greater than 500 cm H_2O/L/s was not tolerated. With resistances between 250 and 500 cm H_2O/L/s, after the initial increase in Pdi, a decrease in Pdi from the highest level reached of 20% or more was observed with loads greater than 350 cm H_2O/L/s. No decrease in Pdi was found with the lesser loads. Therefore, only loads greater than 350 cm H_2O/L/s were used in our studies. The experiments were terminated when Pdi decreased by more than 20% from the highest Pdi reached during loaded breathing. Although blood glucose levels were not monitored, an attempt to minimize the effect of blood glucose level on intramuscular glycogen content was made by allowing all lambs to feed *ad libitum* up to the time of the experiment and by conducting all studies at the same time of day (between 0900 and 1200 h) in a room maintained at 23–25°C.

Preparation for histochemical analysis. All experimental lambs were killed with Euthanasia T61 solution (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ) immediately after terminating the experiment to prevent postexercise glycogen repletion (3, 4, 7). Six additional lambs, aged 2–14 d, served as controls. Tissue samples were obtained from the right or left midcostal area of the diaphragm in the experimental and control lambs. Muscle specimens were frozen in isopentane cooled to its freezing point with liquid nitrogen. Muscle sampling and the freezing processes were completed within 5 min after euthanasia to prevent postmortem changes in intramuscular glycogen content. Although the degree of muscle contraction during biopsy and freezing procedures was not standardized, the effect of muscle contraction on the histochemical analysis of glycogen content has been shown to be minor (30). Samples were stored in liquid nitrogen until they were sectioned. Serial cross sections, 8 μm thick, were cut in a cryostat (Bright/Hacker Instruments, Inc., Fairfield, NJ) at −20°C and were stained for muscle fiber typing and analysis of intramuscular glycogen content. The variability of section thickness with the cryostat is less than 5%. For the analysis of fiber type proportions, more than 700 fibers from each diaphragm specimen were counted, and the percentage for each fiber type was calculated. Histochemically determined glycogen content was quantified in at least 100 fibers for type I and IIa fibers and in at least 50 fibers from type IIc fibers in every lamb.

Myosin ATPase reaction. Muscle fiber typing was performed using the histochemical myosin ATPase reaction at pH 9.4 after alkaline (pH 9.4) or acid (pH 4.5, pH 4.3) preincubation (31, 32). The preincubation pH values were chosen based on work with sheep muscles (32) and were confirmed in our own laboratory after examining a large range of pH (pH 3.9–10.9). This allowed differentiation of fibers into type I, IIa, IIb, and IIc. With the myosin ATPase reaction after alkaline preincubation, type I and type II fibers showed low and high staining density, respectively. With acid preincubation at pH 4.3, staining density of all type I fibers and the majority of the type II fibers was reversed. Some type II fibers maintained moderate or high staining density after acid preincubation, and these were classified as type IIc. When preincubation pH was increased to 4.5, staining density of type II fibers with acid reversal at pH 4.3 remained light and, therefore, these fibers were classified as type IIa (32).

PAS. Intramuscular glycogen content was determined with the PAS reaction (31, 33). The optimal staining conditions for sheep muscle have been studied in our laboratory (1). Based on this study, the stain was performed by fixing in Carnoy's solution for 15 min and incubating in periodic acid for 10 min and in fresh Schiff's reagent (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature (21–23°C). To eliminate the variability in staining technique, sections from control and experimental muscles were stained together. In addition, a section from the same control animal was included with each staining procedure as a standard and the same glycogen content in these standard sections was confirmed. The PAS staining density was quantified as ROD using an image processing system.

Image processing system. The image processing system consisted of a high-resolution charged coupled diode video camera (VSP SC-505, VSP Labs Inc., Ann Arbor, MI) attached to a light microscope (Leitz Dialux 20, Leitz Wetzlar, Germany) and a video monitor (Sony PVM 1271Q, Sony Corporation, Japan). The video camera processes 512-line images at 1 MHz with 100% geometric accuracy. The camera was interfaced with an image processing system that was incorporated into a unix-based multiprocessor super-microcomputer (Masscomp 533; Masscomp, Westford, MA) for digitizing, storing, and analyzing the images. The system was routinely calibrated at 0 and 100% transmitted light. OD measurements with this system are linear over the range of 0.04 to 1.27 as determined with a photographic density step wedge. Because the camera has very low dark currents and excellent signal-to-noise ratio specifications, measurements with our system were highly reproducible (coefficient of variation 1.2% for five measurements taken at 1-min intervals). Shading correction for the system was not necessary because the coefficient of variation of the OD in a uniformly illuminated image divided into 64 equal areas was less than 3%. The video image was composed of 520 × 480 pixels with eight-bit resolution: at a magnification of ×100, one pixel was equal to a diameter of 0.89 μm. In each image taken, the light transmitted through each pixel within an area delineated by a 13 × 13 pixel (12 × 12 μm) square cursor was measured and averaged by the computer. The square cursor was placed in the center of a muscle fiber and covered most of the cross-sectional area of that muscle fiber. The ROD in the square cursor was then calculated from the transmitted light as ROD = $\log_{10}$ (background value/light transmitted value), with background value = 100% (34). Therefore, a higher ROD indicated greater intramuscular glycogen content. Images were taken at ×100 magnification from randomly selected areas of sections, using a light source of 510 nm wavelength. All images were stored within 24 h after cutting and staining to avoid any variability secondary to fading of staining.

Statistical analysis. Values are expressed as mean ± SD. Comparisons of physiologic measurements (Pdi and arterial blood gas tensions) were made using an unpaired t test. Physiologic measurements between baseline and after IFR loads within a group were tested with a paired t test. Statistical analysis of ROD among fiber types or among groups was performed using a one-way analysis of variance with repeated measures and post hoc testing (Duncan multiple-range). Results were considered significant if $p < 0.05$.

RESULTS

Time course of Pdi and Pa_{CO_2} during loaded breathing. Figure 1 shows the time course of Pdi and Pa_{CO_2} in one lamb subjected to a severe IFR load. With this load, Pdi increased rapidly and reached its peak level within 10 min. This high level of Pdi was sustained for a limited period of time, after which it began to decrease continuously until the study was terminated. The increase in Pa_{CO_2} was also rapid. In addition, Pa_{CO_2} began to

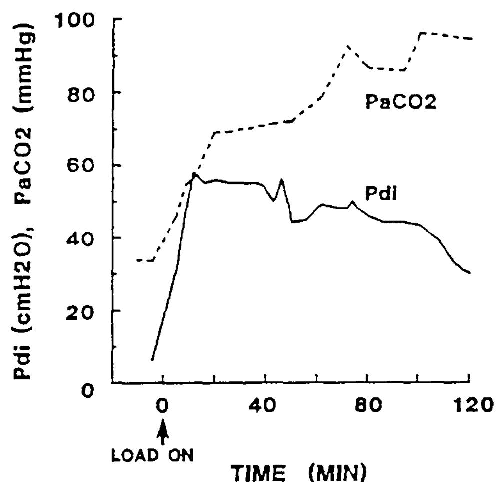

Fig. 1. Time course of Pdi and Pa_{CO_2} in a lamb subjected to a severe IFR load. Note that Pdi and Pa_{CO_2} increased after the load was applied and that Pa_{CO_2} increased further at about the time when Pdi began to decrease.

Table 1. *Changes in Pdi and arterial blood gas tensions in lambs exposed to severe IFR loads**

Duration of loaded breathing (min)	82.0 ± 30.7 (50–121)
Resistance of IFR load (cm H_2O/L/s)	416.7 ± 54.3 (350–480)
Baseline Pdi (cm H_2O)	6.0 ± 2.4 (3.4–10.5)
Highest Pdi at plateau (cm H_2O)	55.0 ± 6.0 (47.5–61.0)†
Baseline Pa_{CO_2}	
kPa	4.8 ± 0.4 (3.1–4.0)
mm Hg	36.3 ± 3.2 (31.8–41.1)
Highest Pa_{CO_2} during loading	
kPa	12.1 ± 2.8 (5.6–11.4)†
mm Hg	91.1 ± 20.9 (56.8–116.3)†
Baseline arterial pH	7.38 ± 0.04 (7.34–7.43)
Lowest arterial pH during loading	7.00 ± 0.12 (6.83–7.19)†
Baseline Pa_{O_2}	
kPa	60.2 ± 9.7 (33.9–53.2)
mm Hg	452.5 ± 73.3 (346.0–542.4)
Lowest Pa_{O_2} during loading	
kPa	21.7 ± 16.7 (5.1–37.7)†
mm Hg	163.2 ± 125.3 (52.0–385.0)†

* Values are expressed as mean ± SD with the range in parentheses.
† $p < 0.005$, loaded breathing compared to baseline value.

increase further at around the time when Pdi began to decrease, reaching about 12.0 kPa (90 mm Hg). This indicated that ventilatory failure occurred. All six lambs studied with severe IFR loads showed the same pattern of Pdi and Pa_{CO_2} (Table 1). The highest Pdi generated with severe IFR loads was nine to 18 times higher than baseline Pdi. At the end of the experiment, Pdi decreased by 28.1 ± 13.1% from peak Pdi. In two lambs, slow and irregular respiration with large fluctuations in Pdi was observed during the last 10 min of the experiment instead of a continuous decrease in Pdi. Although the mean Pa_{O_2} at the end of a study was lower than baseline Pa_{O_2}, in all but one lamb Pa_{O_2} still remained over 11.3 kPa (85 mm Hg), even when a decrease in Pdi and hypercapnea developed. The lamb in whom hypoxia (Pa_{O_2} = 6.9 kPa or 52 mm Hg) was observed at the end of study in spite of oxygen supplementation was a 4-d-old lamb.

Muscle fiber composition in lamb diaphragm. The proportion of each fiber type did not show a correlation with age over the narrow age range examined in our study (2–14 d of age) (r = 0.17 for type I, r = 0.3 for type IIa, and r = 0.39 for type IIc; $p > 0.1$). Thus, fiber type proportions from each lamb were grouped together, and the mean fiber type proportion was calculated. Type I fibers composed 33.4 ± 2.9%, type IIa fibers 61.3 ± 2.6%, and type IIc fibers 5.3 ± 1.1% of the total fibers. There was no difference in fiber type composition between the abdominal side (type I = 34.0 ± 2.4%, type IIa = 61.1 ± 2.7%, and type IIc = 4.9 ± 1.1%) and the thoracic side (type I = 33.9 ± 2.4%, type IIa = 61.1 ± 2.3%, and type IIc = 5.0 ± 1.3%) of the costal diaphragm ($p > 0.1$).

Pattern of glycogen depletion in each fiber type in diaphragm. Figure 2 shows the results for glycogen content in each diaphragmatic fiber type of control and loaded lambs. The glycogen content in the control diaphragm was highest in type IIa (ROD = 0.94 ± 0.06) followed by type IIc (ROD = 0.72 ± 0.10) and type I (ROD = 0.53 ± 0.07). These ROD values were significantly different among the three fiber types ($p < 0.01$). With severe IFR loads, there was a decrease in ROD in all fiber types (type I = 0.12 ± 0.04, type IIa = 0.17 ± 0.08, and type IIc = 0.14 ± 0.09) compared with controls ($p < 0.005$). In addition, the amount of decrease was greatest in type II fibers and least in type I fibers ($p < 0.001$), resulting in similar intramuscular glycogen content among fiber types ($p > 0.1$). To eliminate the possibility that glycogen depletion was related to the surgical procedures performed on the lambs, intramuscular glycogen content in the diaphragm of three instrumented nonloaded lambs was compared with that of noninstrumented lambs. There was no difference in the glycogen content in each of the diaphragmatic fiber types between instrumented (type I = 0.54 ± 0.07, type IIa = 0.91 ± 0.03, and type IIc = 0.76 ± 0.15) and noninstrumented (type I = 0.53 ± 0.09, type IIa = 0.96 ± 0.09, and type IIc = 0.67 ± 0.06) lambs.

DISCUSSION

Although there have been previous studies that have explored the relation of glycogen use in the diaphragm to increased work loads, our study differs from earlier experiments in several significant ways. This study is, to our knowledge, the first to report glycogen use by the diaphragm of the awake, loaded neonatal animal without the confounding effects of surgery and anesthesia. Most previous studies have examined this issue in the adult (1–10, 35). Recently, Mayock *et al.* (36) investigated glycogen use in 1-mo-old piglets subjected to moderate IFR loads, but their animals were anesthetized. Unlike many studies in which the work of breathing was increased by swimming or treadmill exercise (3–6, 9, 10, 35), the work load in our experiments was directly targeted to the diaphragm by use of IFR loads. Also,

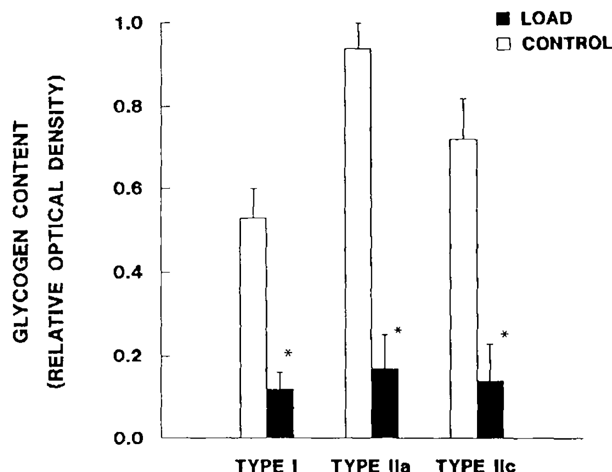

Fig. 2. Mean glycogen content expressed as ROD in the costal diaphragm from control lambs and experimental lambs subjected to severe IFR loads. With these loads, glycogen content decreased in all fiber types, with the decrease being greatest in type IIa fibers, followed by type IIc and type I fibers. *, $p < 0.005$, loaded compared with control for each fiber type.

glycogen content was objectively analyzed in individual fibers by use of a computerized densitometric technique (30). This method has several advantages over previously used methods in which glycogen was measured either biochemically in muscle homogenates (2, 3, 5, 7–9, 35, 36) or by use of a qualitative, subjective rating of the intensity of the staining reaction (1, 6). The first advantage is that glycogen content can be measured in single muscle fibers, thereby allowing the evaluation of the selectivity of glycogen use by metabolically different fiber types. This method also allows the assessment of regional differences in content within a given fiber. Because the distribution of glycogen was homogeneous within the diaphragmatic fibers, we used a cursor size that encompassed the whole fiber (except for a few peripheral regions caused by the square shape of the cursor *versus* the circular or ellipsoid shape of the muscle fiber) and obtained an average content within a fiber. A second advantage is that the measurements are made by a computer using a standardized optical calibration procedure. This eliminates the variability that might be introduced by using a subjective rating system. A third advantage is that the optical measurements of a properly performed PAS reaction correlate well with biochemically determined levels of glycogen (30). Therefore, the data represent a quantitative rather than a qualitative assessment of glycogen content. Finally, because the measurements are made from images of the section rather than from the section itself, there is no variability introduced from potential fading of staining with time as the analysis of a section is being performed, a process that may extend over several hours depending on the number of fibers being analyzed.

One of the main findings in our study is that intramuscular glycogen is consumed in large quantities in the neonatal diaphragm during increased energy requirements. Our results, therefore, refute the hypothesis that there is a major limitation of glycogen use in the neonatal diaphragm. This finding was not expected, inasmuch as previous studies have shown that glycogenolytic and glycolytic enzymes such as glycogen phosphorylase, phosphorylase kinase, lactate dehydrogenase, and phosphofructokinase are low in neonatal skeletal muscles and young rat diaphragm (15–25). This apparent discrepancy could be explained by differences in maturation between species. Developmental changes in glycogenolytic and glycolytic enzyme activities have been studied mainly in rodents, such as the rat, whose muscles are poorly differentiated at birth (37) and are probably less mature than newborn lamb muscles.

The depletion of glycogen in the neonatal lamb diaphragm was found to occur at the time when the diaphragm was unable to maintain force generation with high loads. Because oxidative metabolism and use of other energy substrates were not measured in this study, we cannot conclude that the depletion of intramuscular glycogen induced the decrease in diaphragmatic force. Also, the role of glycogen depletion as a cause for loss of force generating capacity has been questioned (38, 39). Therefore, our results show that intramuscular glycogen is one important energy source for diaphragmatic function, but we do not suggest an association between force loss and glycogen depletion. Interestingly, our finding contrasts with the recent results of Mayock *et al.* (36), who used anesthetized young piglets. Despite a relatively large load that induced about six times the baseline Pdi, no glycogen depletion was observed in Mayock's study. At present, we have no direct explanation for the lack of glycogen use in their study, although it might be related to species difference and anesthesia.

Another important finding in our study is that the pattern of glycogen depletion in the neonatal diaphragm with IFR loads depends on fiber type. Although all fiber types used intramuscular glycogen during loaded breathing, glycogen depletion with severe loads was greater in type IIa than in type I and IIc fibers. This finding suggests that either *1*) type IIa fibers in the lamb diaphragm have a greater glycolytic capacity than other fiber types or *2*) glycogen in type I fibers was spared because substrates other than intramuscular glycogen are primarily used for energy. Furthermore, large amounts of glycogen were depleted from type IIa fibers, which in the neonatal diaphragm compose a proportion of total fibers higher than that in the adult diaphragm in several species (11–14) including the sheep (unpublished data from our laboratory). Based on this finding, we speculate that the newborn lamb may be more dependent on intramuscular glycogen as an important energy substrate than the adult, especially when subjected to severe loads.

In summary, we have shown that, in unanesthetized neonatal lambs breathing through IFR loads, glycogen content in the costal diaphragm decreased with loaded breathing. In addition, marked depletion in glycogen content was seen at the time when there was a decrease in diaphragmatic force. The reduction in glycogen content occurred in all fiber types, and the depletion of glycogen with severe IFR loads was greater in type IIa fibers than in other fiber types.

Acknowledgments. The authors thank Dr. Gary C. Sieck for helpful discussions and guidance in performing the histochemical procedures and Dr. Gabriel G. Haddad for ongoing discussions and critical review of the manuscript. We also thank Ted Cummins for his help with the image processing system and Ralph Garcia for technical assistance.

REFERENCES

1. Bazzy AR, Akabas SR, Hays AP, Haddad GG 1988 Respiratory muscle response to load and glycogen content in type I and II fibers. Exp Neurol 101:17–28
2. Ferguson GT, Irvin CG, Cherniack RM 1990 Relationship of diaphragm glycogen, lactate, and function to respiratory failure. Am Rev Respir Dis 141:926–932
3. Górski J, Namiot Z, Giedrojć J 1978 Effect of exercise on metabolism of glycogen and triglycerides in the respiratory muscles. Pflugers Arch 377: 251–254
4. Green HJ, Ball-Burnett ME, Morrissey MA, Spalding MJ, Hughson RL, Fraser IG 1987 Fiber type specific glycogen utilization in rat diaphragm during treadmill exercise. J Appl Physiol 63:75–83
5. Green HJ, Ball-Burnett ME, Morrissey MA, Kile J, Abraham GC 1988 Glycogen utilization in rat respiratory muscles during intense running. Can J Physiol Pharmacol 66:917–923
6. Ianuzzo CD, Spalding MJ, Williams H 1987 Exercise-induced glycogen utilization by the respiratory muscles. J Appl Physiol 62:1405–1409
7. Lockhat D, Roussos C, Ianuzzo CD 1988 Metabolic changes in the loaded hypoperfused and failing diaphragm. J Appl Physiol 65:1563–1571
8. Namiot Z, Giedrojć J, Górski J 1985 The effect of increased respiratory resistance on glycogen and triglyceride levels in the respiratory muscles of the rat. Eur J Appl Physiol 54:432–435
9. Namiot Z, Górski J 1988 Regulation of glycogen metabolism in rat respiratory muscles during exercise. Eur J Appl Physiol 58:120–124
10. Reid WD, Cairns C, McRae D, Yip M, Pardy RL 1989 Regional and fibre type glycogen depletion patterns of the hamster diaphragm following submaximal and maximal swimming. Am Rev Respir Dis 139:A166(abstr)
11. Keens TG, Bryan AC, Levison H, Ianuzzo CD 1978 Developmental pattern of muscle fiber types in human ventilatory muscles. J Appl Physiol 44: 909–913
12. Le Souëf PN, England SJ, Stogryn HAF, Bryan AC 1988 Comparison of diaphragmatic fatigue in newborn and older rabbits. J Appl Physiol 65: 1040–1044
13. Mayock DE, Hall J, Watchko JF, Standaert TA, Woodrum DE 1987 Diaphragmatic muscle fiber type development in swine. Pediatr Res 22:449–454
14. Smith D, Green H, Thomson J, Sharratt M 1988 Oxidative potential in developing rat diaphragm, EDL, and soleus muscle fibers. Am J Physiol 254: C661–C668
15. Baldwin KM, Hooker AM, Campbell PJ, Lewis RE 1978 Enzyme changes in neonatal skeletal muscle: effect of thyroid deficiency. Am J Physiol 235: C97–C102
16. Colling-Saltin A-S 1978 Some quantitative biochemical evaluations of developing skeletal muscles in the human foetus. J Neurol Sci 39:187–198
17. Hudlická O, Pette D, Staudte H 1973 The relation between blood flow and enzymatic activities in slow and fast muscles during development. Pflugers Arch 343:341–356
18. Nemeth PM, Norris BJ, Solanki L, Kelly AM 1989 Metabolic specialization in fast and slow muscle fibers of the developing rat. J Neurosci 9: 2336–2343
19. Smith PB 1980 Postnatal development of glycogen- and cyclic AMP-metabolizing enzymes in mammalian skeletal muscle. Biochim Biophys Acta 628: 19–25
20. Powers S, Lawler J, Criswell D, Dodd S, Silverman H 1990 Age related enzymatic changes in the rat diaphragm. FASEB J 4:A292(abstr)

21. Gross SR, Bromwell K 1977 Postnatal development of phosphorylase kinase in mouse skeletal mouse. Arch Biochem Biophys 184:1–11
22. Novák E, Drummond GI, Skála J, Hahn P 1972 Developmental changes in cyclic AMP, protein kinase, phosphorylase kinase, and phosphorylase in liver, heart, and skeletal muscle of the rat. Arch Biochem Biophys 150: 511–518
23. Richter F, Böhme H-J, Hofmann E 1988 Changes of glycogen phosphorylase isozyme pattern in rat tissues during pre- and postnatal development. Biomed Biochim Acta 47:743–752
24. Villa-Moruzzi E, Locci-Cubeddu T, Bergamini E 1979 Developmental changes of glycogen enzymes in fast and slow muscles of the rat. Growth 43:73–79
25. Villa-Moruzzi E 1985 Phosphorylase phosphatase and phosphatase activating-kinase F_A in growing rat muscles. Growth 49:417–425
26. Asotra K, Malhotra RK 1978 Growth metabolism of diaphragm in Swiss albino mice. Experientia 34:75–77
27. Dall'Aglio E, Chang H, Reaven GM, Azhar S 1987 Age-related changes in rat muscle glycogen synthase activity. J Gerontol 42:168–172
28. Dawes GS, Shelley HJ 1968 Physiological aspects of carbohydrate metabolism in the foetus and newborn. In: Dickens F, Randle PJ, Whelan WJ (eds) Carbohydrate Metabolism and Its Disorders. Academic Press, London, pp 87–121
29. Tomanek RJ 1975 A histochemical study of postnatal differentiation of skeletal muscle with reference to functional overload. Dev Biol 42:305–314
30. Halkjær-Kristensen J, Ingemann-Hansen T 1979 Microphotometric determination of glycogen in single fibres of human quadriceps muscle. Histochem J 11:629–638
31. Dubowitz V, Brooke MH (eds) 1973 Histological and histochemical stains and reactions. In: Muscle Biopsy: A Modern Approach. WB Saunders, Philadelphia, pp 20–33
32. Suzuki A 1976 The pH stability of myofibrillar adenosine triphosphatase of five fiber types in skeletal muscles of the sheep and cattle. Jpn J Zootech Sci 47:95–103
33. Pearse AGE 1968 Histochemistry-Theoretical and Applied. Little, Brown and Co, Boston, pp 307–322
34. Choca JI, Clark FM, Green RD, Proudfit HK 1988 A low-priced computer-assisted densitometer and analysis software for quantitative autoradiography. J Neurosci Methods 24:145–154
35. Fregosi RF, Dempsey JA 1986 Effects of exercise in normoxia and acute hypoxia on respiratory muscle metabolites. J Appl Physiol 60:1274–1283
36. Mayock DE, Standaert TA, Woodrum DE 1990 Combined effect of inspiratory resistive loaded breathing and hypoxemia on diaphragmatic force generation and substrate availability in the piglet. Am Rev Respir Dis 141:A368(abstr)
37. Dubowitz V 1965 Enzyme histochemistry of skeletal muscle. Part I. Developing animal muscle. J Neurol Neurosurg Psychiatry 28:516–519
38. Bigland-Ritchie B, Cafarelli E, Vollestad NK 1986 Fatigue of submaximal static contractions. Acta Physiol Scand 128(suppl 556):137–148
39. Vollestad NK, Sejersted OM 1988 Biochemical correlates of fatigue: a brief review. Eur J Appl Physiol 57:336–347