

Effect of growth and training on muscle adaptation in Thoroughbred horses

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Objective—To determine the effect of growth and training on metabolic properties in muscle fibers of the gluteus medius muscle in adolescent Thoroughbred horses.

Animals—Twenty 2-year-old Thoroughbreds.

Procedure—Horses were randomly assigned to 2 groups. Horses in the training group were trained for 16 weeks, and control horses were kept on pasture without training. Samples were obtained by use of a needle-biopsy technique from the middle gluteus muscle of each horse before and after the training period. Composition and oxidative enzyme (succinic dehydrogenase [SDH]) activity of each fiber type were determined by use of quantitative histochemical staining procedures. Whole-muscle activity of SDH and glycolytic enzyme (phosphofructokinase) as well as myosin heavy-chain isoforms were analyzed biochemically and electrophoretically, respectively.

Results—The SDH activity of type-I and -IIA fibers increased during growth, whereas whole-muscle activity was unchanged. Percentage of type-IIX/B muscle fibers decreased during training, whereas that of myosin heavy-chain IIA increased. The SDH activity of each fiber type as well as whole-muscle SDH activity increased during training. An especially noticeable increase in SDH activity was found in type-IIX/B fibers.

Conclusions and Clinical Relevance—Changes in muscle fibers of adolescent Thoroughbreds are caused by training and not by growth. The most noticeable change was for the SDH activity of type-IIX/B fibers. These changes in the gluteus medius muscle of adolescent Thoroughbreds were considered to be appropriate adaptations to running middle distances at high speeds. (*Am J Vet Res* 2002;63:1408–1412)

The effects of training on skeletal muscles have been examined in several species of animals by use of histochemical and biochemical analysis.¹⁻⁴ The high trainability of oxidative enzymatic activity in muscles of horses has been extensively documented.⁵⁻¹¹ Roneus et al¹² reported that citrate synthase and 3-hydroxyacyl-CoA dehydrogenase activity doubled during several months of endurance training. Serrano et al¹³ documented that endurance training induces a reversible transition in

myosin heavy-chain (MHC) composition between type-IIa and -I as well as type-IIx and -IIa fibers. Natural growth also appears to induce an increase in activity of muscle oxidative enzymes in horses and other animals. For example, Rivero et al¹⁴ indicated that there was an increase in the percentage of type-I fibers and concomitant decrease in the percentage of type-IIB fibers during growth in male and female Andalusian and Arabian horses. To our knowledge, few studies have considered differential muscle adaptation to growth and training in muscles of horses.

In the study reported here, quantitative analysis of the activity of an oxidative enzyme (succinic dehydrogenase [SDH]) was used to determine the adaptation of muscle fibers to growth alone and to growth plus training. Thoroughbreds were selected because of the long history of managed breeding programs, and this breed of horse has an outstanding inherited ability to run. Therefore, we considered that use of Thoroughbreds to conduct research on muscle adaptation to growth and training would provide basic information for physical fitness training.

Materials and Methods

Animals—Twenty adolescent (2 years old) Thoroughbreds that weighed (mean \pm SD) 449 \pm 30 kg (range, 413 to 515 kg) were used in the study. Before the experiment, all horses were accustomed to handling and riding procedures. All horses received concentrate twice daily, and water and hay were available ad libitum throughout the study. All procedures used in this study were approved by the Animal Experiment Committee of the Equine Research Institute, Tochigi Branch, Japan.

Training procedure—Horses were randomly allocated to 2 groups. Ten horses comprised the training group. The remaining 10 horses served as a control group. During the study, the control group was kept on pastures and allowed to move about freely.

Horses in the training group were trained for 16 weeks. During the initial 3 weeks, horses ran 3 d/wk at a speed of 600 m/min for a distance of 1,600 m and 2 d/wk at a speed of 600 m/min for a distance of 2,400 to 3,200 m. During weeks 4 to 10, horses ran 3 d/wk at a speed of 600 to 800 m/min for a distance of 1,600 m and 2 d/wk at a speed of 600 m/min for a distance of 2,400 to 3,200 m. During weeks 11 to 16, horses ran 3 d/wk at a speed of 800 to 1,000 m/min for a distance of 1,600 m and 2 d/wk at a speed of 600 m/min for a distance of 2,400 to 3,200 m. On days on which the horses did not train, they were kept in a pasture.

Veterinarians monitored health of the horses during the training period. All horses tolerated the 16-week training period well, and none had indications of musculoskeletal disorders. After the training period, mean weight of horses in the control and training groups was 458 \pm 38 kg (range, 415 to 538 kg).

Collection of muscle samples—Samples were obtained from all horses from the same portion of the middle gluteus

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muscle and at the same depth (5 cm from the skin surface) before and after the training period. Samples were frozen in melting isopentane cooled by liquid nitrogen, and they then were stored at -80°C until analyzed. All the samples were analyzed at the same time by use of histochemical or biochemical procedures.

Histochemical analysis of muscle samples—Frozen pieces of muscle were cut with a freezing microtome^a into 3 transverse sections (thickness of each section, 10 μm). Two of the sections were used to detect myofibrillar ATPase.¹⁵ One section was incubated at pH 4.6, and the other was incubated at pH 10.3. After incubation, these sections were histochemically stained for myofibrillar ATPase. The third section was stained to detect SDH activity.¹⁶ This section was incubated for 8 minutes in phosphate buffer (pH, 7.6) containing sodium succinic and **nitro-blue tetrazolium (NBT)**. The reduction of NBT to NBT-diformazan was used as the indicator for the reaction. Microscopic images of muscle fibers were obtained by use of a personal computer and image-processing system.^b During the analysis, optical intensity was maintained constant. To measure the concentration of stain in muscle fibers, luminosity was expressed at 256 Gy. Luminosity was calibrated by use of 4 filters with differing transmissivity (100, 25, 6, and 1.5%).

On the basis of examination of the stained images, muscle fibers were classified as type-I, -IIA, and -IIX/B fibers. Then, composition of muscle fibers (ie, percentage of each fiber type) was calculated for approximately 500 muscle fibers by use of an image-processing system.^b At least 25 areas were measured to determine each fiber type as well as SDH activity. Concentration of NBT-diformazan was calculated on the basis of optical density measured at 570 nm (OD_{570}) by use of the equation of Beer-Lambert, and mean SDH activity of each fiber was expressed as millimoles of fumarate per liter per minute as determined by use of the following equation:

$$\text{concentration of NBT-diformazan} = \text{OD}_{570}/(k \cdot l)$$

where k is the molar extinction coefficient for NBT-diformazan (ie, 26,478/mol/cm), and l is the path length for light absorbance (ie, 10 μm). Furthermore, on the basis of muscle

fiber composition and area, the contribution (percentage) of each fiber type to total area was calculated.

Biochemical analysis of muscle samples—Activities of 2 enzymes (SDH and **phosphofructokinase [PFK]**) were used to determine the oxidative and glycolytic capacities, respectively, of muscle samples. All enzyme assays were performed spectrophotometrically^c in triplicate at 25°C , and results were expressed as number of micromoles per gram of noncollagenous protein per minute. Protein concentrations were determined by use of a protein assay kit^d that used bovine albumin as the standard.

For each horse, 1 piece of muscle was homogenized in ice-cold 33.3mM phosphate buffer (pH 7.4). The SDH activity was determined on the basis of the technique of Cooperstein et al.¹⁷ The electronic transfer system was blocked by the addition of cyanogen, and the extent of the reduction in cytochrome C was determined from the change in absorbance measured at 550 nm. Another piece of muscle was homogenized in ice-cold homogenization medium containing 150mM KCl, 50mM KHCO_3 , and 6mM EDTA. The PFK activity was evaluated on the basis of the technique of Shonk and Boxer.¹⁸

Quantification of MHC isoforms—To analyze the expression of MHC isoforms, muscle pieces were homogenized in 10 volumes of ice-cold buffer solution containing 0.1M KCl, 1mM EDTA, and 20mM tris maleate buffer (pH, 7.0). Homogenate was centrifuged ($1,000 \times g$ for 10 minutes at 4°C), and the pellet again was homogenized (1:40 [wt:vol]) in SDS buffer solution containing 30% (vol:vol) glycerol, 5% (vol:vol) β -mercaptoethanol, 2.3% (wt:vol) SDS buffer solution, 62.5mM tris-HCl (pH 6.8), and 0.05% (wt:vol) bromophenol blue. Homogenate was incubated for 10 minutes at 60°C and then further diluted 1:25 with the same buffer solution. A 7.5- μL portion of the homogenate then was electrophoresed.

Analysis of MHC isoforms was electrophoretically performed as described elsewhere.^{19,20} Briefly, SDS-PAGE was performed on a slab gel (7 cm \times 9 cm \times 1 mm) by use of a 7 to 10% (wt:vol) polyacrylamide-30 to 40% (vol:vol) glycerol gradient separating gel and a 3.5% (wt:vol) poly-

Table 1—Effect of growth and training on histochemical and biochemical properties in the gluteus medius muscle fibers obtained from twenty 2-year-old Thoroughbreds (10 control horses and 10 horses subjected to training) before and after a 16-week period of training

| Variable | Control | | | Training | | |
|---|------------------|-------------------|---------|-------------------|-------------------|---------|
| | Before | After | P value | Before | After | P value |
| Muscle fiber composition (%) | | | | | | |
| I | 17.8 \pm 6.5 | 24.5 \pm 5.8 | 0.09 | 12.5 \pm 4.2 | 11.7 \pm 5.0 | 0.68 |
| IIA | 45.8 \pm 4.5 | 43.9 \pm 7.7 | 0.88 | 40.4 \pm 7.6 | 47.3 \pm 5.1 | 0.09 |
| IIX/B | 36.4 \pm 6.4 | 31.6 \pm 6.8 | 0.07 | 47.1 \pm 4.9 | 41.0 \pm 4.0 | 0.01 |
| Muscle fiber area (μm^2) | | | | | | |
| I | 1,952 \pm 455 | 1,960 \pm 347 | 0.77 | 1,909 \pm 414 | 2,227 \pm 261 | 0.06 |
| IIA | 2,290 \pm 385 | 2,277 \pm 405 | 0.89 | 2,411 \pm 502 | 2,704 \pm 721 | 0.06 |
| IIX/B | 3,841 \pm 648 | 3,920 \pm 620 | 0.76 | 4,294 \pm 794 | 4,577 \pm 500 | 0.13 |
| Relative contribution of fiber type to total area (%) | | | | | | |
| I | 12.8 \pm 5.9 | 17.7 \pm 5.4 | 0.31 | 7.4 \pm 3.1 | 7.6 \pm 3.1 | 0.40 |
| IIA | 37.4 \pm 5.3 | 36.7 \pm 9.1 | 0.59 | 30.2 \pm 6.2 | 37.1 \pm 7.2 | 0.07 |
| IIX/B | 49.8 \pm 9.1 | 45.6 \pm 12.1 | 0.74 | 62.4 \pm 5.5 | 55.3 \pm 7.5 | 0.02 |
| Myosin heavy-chain isoform composition (%) | | | | | | |
| I | 14.9 \pm 6.0 | 10.8 \pm 3.4 | 0.31 | 14.5 \pm 3.4 | 14.3 \pm 5.1 | 0.88 |
| Ila | 33.7 \pm 4.0 | 30.2 \pm 4.5 | 0.01 | 40.7 \pm 4.1 | 44.8 \pm 6.6 | 0.01 |
| IIX and IIb | 51.5 \pm 8.0 | 59.0 \pm 6.5 | 0.41 | 44.8 \pm 3.8 | 42.7 \pm 7.7 | 0.07 |
| Enzyme activity ($\mu\text{mol/g}$ of NCP/min) | | | | | | |
| SDH | 62.3 \pm 19.2 | 66.9 \pm 25.2 | 0.30 | 60.1 \pm 22.2 | 82.4 \pm 46.8 | 0.05 |
| PFK | 422.2 \pm 65.8 | 355.2 \pm 105.8 | 0.15 | 491.3 \pm 201.8 | 576.3 \pm 271.9 | 0.04 |

Values reported are mean \pm SD.
NCP = Noncollagenous protein. SDH = Succinic dehydrogenase. PFK = Phosphofructokinase.

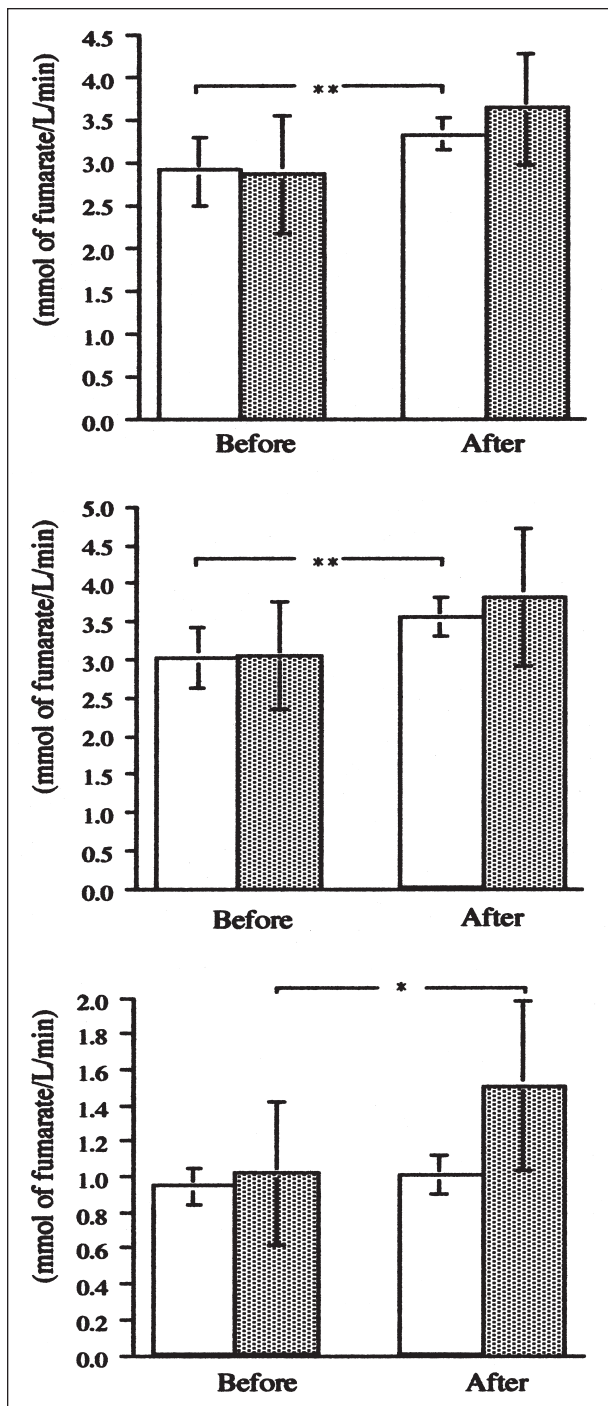


Figure 1—Quantitative analysis of mean \pm SD succinic dehydrogenase activity for type-I (top), -IIA (middle), and -IIX/B (bottom) muscle fibers in samples of the middle gluteus muscle obtained from twenty 2-year-old Thoroughbreds (10 control horses [white bars] and 10 horses subjected to training [gray bars]) before and after a 16-week period of training. *Values differ significantly ($P < 0.05$) **Values differ significantly ($P = 0.01$).

acrylamide stacking gel containing 35% (vol:vol) glycerol. Electrophoresis was initially 60 V/plate until the tracking dye completely entered the separating gel, and it then was 150 V/plate for 22 hours at 12°C. Gels were stained by use of a silver staining kit.^c Composition of MHC isoforms was calculated by use of a scanner equipped with a laser attachment.^f

Statistical analysis—Values were reported as mean \pm SD. All values were analyzed by use of a paired *t* test within each group to detect the effect of training or growth. In all cases, significance was established at values of $P < 0.05$.

Results

Muscle fiber composition and area—In the control group, muscle fiber composition was characterized by an increase in type-I fibers and a decrease in type-IIX/B fibers (Table 1). In contrast, the percentage of type-IIX/B fibers in horses after training decreased significantly, compared with the percentage before training. In trained horses, muscle fiber composition was characterized by an increase in type-IIA fibers.

We did not detect a change in muscle fiber area in the control horses during growth for any type of muscle fiber (Table 1). However, muscle fiber area increased slightly for type-I and -IIA fibers after training, compared with the value before training.

We did not detect a change in relative contribution of any type of fiber in the control horses during growth (Table 1). In contrast, the relative contribution of type-IIX/B fibers decreased significantly after training, compared with the value obtained before training. There was an increase in the relative contribution for type-IIA fibers in horses after training.

MHC isoforms—Although there are few MHC-IIb isoforms in horses, all of the adult types of MHC isoforms were detected in the samples obtained from the middle gluteus muscle. In the control group, the composition of MHC-IIa isoforms decreased during growth (Table 1). In contrast, the composition of MHC-IIa isoforms increased significantly after training, and the composition of MHC-IIx and -IIb isoforms typically decreased after training, compared with values before training.

Enzymatic activity—The SDH or PFK activity did not change in the control horses during growth (Table 1). In contrast, activity of SDH and PFK increased significantly after training, compared with values before training.

With regard to SDH activity of each type of fiber, we did not detect a change in the SDH activity of type-IIX/B fibers in control horses during growth; however, the SDH activity of type-I and -IIA fibers increased significantly in control horses during growth (Fig 1). In contrast, SDH activity of type-IIX/B fibers increased significantly after training, compared with SDH activity before training. The SDH activity increased in type-I and -IIA fibers.

Discussion

In general, it requires 5 years for a Thoroughbred to complete its growth. During the first year, horses reach approximately 65% of adult body weight and 90% of adult height; subsequently, body length and width continue to increase.^{21,22} The horses used in the study reported here were at the beginning of the second phase of growth. This period is critical for Thoroughbreds, because it is the time when full-scale training begins. The purpose of the study reported here was to determine changes in muscle fibers attributable

to growth and training, respectively. The most noticeable change during growth was the increase in SDH activity of type-I and -IIA fibers, whereas the most noticeable change during training was the increase in SDH activity of type-IIX/B fibers as well as whole-muscle SDH activity.

In other studies,²³⁻²⁶ investigators have used immunohistochemical and electrophoretic methods to document that type-IIB fibers in horses should be termed type-IIX fibers, as determined on the basis of traditional myofibrillar ATPase histochemical analysis. In addition, a large amount of MHC-IIx isoform and a small amount of MHC-IIb isoform were detected by use of electrophoretic methods in the study reported here. Therefore, although the muscle fiber types were classified on the basis of traditional histochemical analysis, we used the term type-IIX/B fiber instead of type-IIB fiber for this study.

Histochemical analysis of samples from the control group revealed that the contribution of type-I muscle fiber to total area increased by 5%. A quantitative increase in type-I muscle fiber during growth can be found in many mammals and appears to be more evident in larger animals.²⁷ Furthermore, it has been reported¹⁴ for racing horses that the amount of type-I fibers increases gradually from 1 to 10 years of age in Andalusian and Arabian horses. It is believed that the quantitative increase supports the increase in body weight during growth.

Biochemical analysis of samples from the control group did not reveal growth-related changes in whole-muscle PFK activity. This result is consistent with other studies^{28,29} in which investigators documented that skeletal muscle of horses essentially has a high capacity for glycolysis and that the activity does not increase during growth. Consistent with results of the quantitative analysis of the control group, the increase in whole-muscle SDH activity is mainly dependent on the increase of type-I and -IIA muscle fibers. In addition to the quantitative increase in type-I muscle fibers, an improvement in the aerobic energy supply system seems to be an important growth-related change necessary to support a heavier body.

The net effect of training should be represented by the difference of change between the control and training groups. On the basis of this concept, the following 3 points were considered to be the main effects attributable to training in this study. First, there was an increase in the expression of MHC-IIa isoform. Second, we detected an increase in whole-muscle SDH activity that was accompanied by an increase in SDH activity for type-IIX/B fibers. Finally, there was an increase in whole-muscle PFK activity.

After the 16-week training period, the amount of MHC-IIa isoform increased significantly with concomitant decreases in MHC-IIx and -IIb isoforms and an increase in whole-muscle SDH activity. This result is consistent with the histochemical analysis that revealed decreases in the muscle composition and contribution of type-IIX/B fibers. In several species of mammals, endurance training increases the activity of oxidative enzymes and the percentage of high oxidative fibers. For example, Snow and Guy⁵ reported

approximately a 2-fold increase in oxidative and glycolytic enzymatic activities and a transition toward a more oxidative muscle fiber during training. In that study, training consisted of long-distance (trotting and cantering for a distance of 10 to 15 km) and high-speed (galloping for a distance of 600 m 3 times) training each day. Lovell and Rose³⁰ also revealed a significant increase in the percentage of type-IIA fibers, with approximately a 1.5-fold increase in the activity of oxidative and glycolytic enzymes following a combination of high-intensity (90 to 100% maximal heart rate) interval and long-distance training events. Furthermore, Serrano et al¹³ clearly documented an increase in expression of MHC-IIa isoform with a concomitant decrease in expression of MHC-IIx isoform after 3 months of endurance training. It should be mentioned that the increase in SDH activity in the study reported here was mainly dependent on the increase in activity among type-IIX/B fibers. Because type-IIX/B fibers provide the greatest contribution to total muscle volume, the increase in SDH activity in type-IIX/B fibers would markedly affect the increase in whole-muscle SDH activity. The remarkable improvement in the oxidative capacity of muscle during training depended on the 1.5-fold increase in the SDH activity of type-IIX/B fibers.

Analysis of data for muscles obtained from the hind limbs of cats has revealed that only type-I and -IIA fibers are recruited at slow running speeds and that type-IIX and -IIX/B fibers are recruited at high running speeds or for jumping.³¹ Concerning the gluteus medius muscle of horses, there is insufficient data to clearly describe the order of recruitment of each fiber type. To efficiently stimulate type-IIX/B fibers, information is needed on the recruitment order and the critical amount and type of exercise needed for recruitment of each fiber type.

^a5030, Bright Instrument, Huntingdon, England.

^bLeica DC 100, Leica, Tokyo, Japan.

^cJASCO UVIDEK-660, Japan Spectroscopic Co, Tokyo, Japan.

^dProtein assay kit, Bio-Rad Laboratories, Hercules, Calif.

^eSilver staining kit, Wako Pure Chemicals Co, Osaka, Japan.

^fDensitograph, ATTO Co, Tokyo, Japan.

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