

Properties of slow- and fast-twitch skeletal muscle from mice with an inherited capacity for hypoxic exercise

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Abstract

Muscle fiber type, myosin heavy chain (MHC) isoform composition, capillary density (CD) and citrate synthase (CS) activity were investigated in predominantly slow-twitch (soleus or SOL) and fast-twitch (extensor digitorum longus or EDL) skeletal muscle from mice with inherited differences in hypoxic exercise tolerance. Striking differences in hypoxic exercise tolerance previously have been found in two inbred strains of mice, Balb/cByJ (C) and C57BL/6J (B6), and their F1 hybrid following exposure to hypobaric hypoxia. Mice from the three strains were exposed for 8 weeks to either normobaric normoxia or hypobaric hypoxia (1/2 atm). Hypoxia exposure led to a slightly higher 2b fiber composition and a lower fiber area of types 1 and 2a in SOL of all mice. In the EDL, muscle fiber and MHC isoform composition remained unaffected by chronic hypoxia. Chronic hypoxia did not significantly affect CD in either muscle from any of the three strains. There were relatively larger differences in CS activity among strains and treatment, and in SOL the highest CS activity was found in the F1 mice that had been acclimated to hypoxia. In general, however, neither differences among strains nor treatment in these properties of muscle vary in a way that clearly relates to inherited hypoxic exercise tolerance.

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1. Introduction

The relationship between physiological properties that promote a high capacity for endurance exercise and those that accompany chronic exposure to environmental hypoxia has long been of interest to physiologists (Hochachka et al., 1998). This connection is conceptually satisfying because of the presumption that both endurance exercise and chronic hypoxia impose similar demands for an enhanced capacity for oxygen delivery to muscle and improved contractile economy of muscle. It has even been proposed that the physiological traits associated with endurance capability and hypoxia tolerance co-evolved in humans, and these characteristics have been formally integrated in a ‘hypoxia tolerance/endurance exercise phenotype’ (Hochachka et al., 1998, 1999). In this context, it has been suggested that skeletal muscle with a

high proportion of slow-twitch fibers (type 1), a high capillary density (CD) and an increased reliance on aerobic metabolism is ideally suited for both endurance exercise and environmental hypoxia (Hochachka et al., 1998).

The effects of endurance training on skeletal muscle structural–functional properties are well characterized, and training typically leads to an increased proportion of oxidative fiber types and a higher oxidative metabolic capacity (reviewed in Booth and Baldwin, 1996), as well as an increased CD (Anderson and Henriksson, 1977; Bigard et al., 1991; Brodal et al., 1977; Saltin and Gollnick, 1983). There is less evidence, however, for such changes in skeletal muscle as a result of chronic exposure to environmental hypoxia. Fiber-type transformations were not seen in skeletal muscle from rat (Ishihara et al., 1995; Sillau and Banchemo, 1977; Takahashi et al., 1992), guinea pig (Jackson et al., 1987; Sillau et al., 1980) or humans (Green et al., 1989; Saltin et al., 1980) following exposure to hypoxia. Where fiber type transitions have occurred in response to hypoxia, the trend has been toward an increase

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in fast-twitch fibers (type 2) or type 2 myosin heavy chains (MHC) (Bigard et al., 1991; Jackson et al., 1987; Ishihara et al., 1995; Mortola and Naso, 1995). Muscle oxidative capacity typically decreases in skeletal muscle following chronic exposure to hypoxia (Green et al., 1989; Howald et al., 1990; Hoppeler and Vogt, 2001; MacDougall et al., 1991), which is also contrary to the response following endurance training but consistent with the change to faster muscle fiber types. Studies of the effect of hypoxia on capillarity in skeletal muscle have failed to yield a consensus. While several reports have found that chronic hypoxia does not induce changes in CD or capillary-to-fiber ratios (Bigard et al., 1991; Mathieu-Costello, 1989; Poole and Mathieu-Costello, 1989, 1990; Sillau and Bancho, 1979; Sillau et al., 1980; Snyder et al., 1985), other studies have found that capillarity or arteriolar density increases upon hypoxic exposure (Boutellier et al., 1983; Deveci et al., 2001; Hoppeler et al., 1990; Smith and Marshall, 1999).

The available data, therefore suggest that in mammalian skeletal muscle, some of the traits that are beneficial for endurance exercise (slow-twitch fibers, high CD and high oxidative capacity) do not confer an advantage under conditions of chronic environmental hypoxia (and vice versa). However, it might be expected that when making comparisons among individuals that have been acclimated to hypoxia, a high capacity for endurance performance *under hypoxia* is associated with a phenotype that is analogous to that of an endurance athlete at sea-level. To address this issue, we investigated the effects of chronic hypobaric hypoxia on skeletal muscle fiber type, MHC composition, capillarity, and oxidative potential in mice in the context of a known genetic model system of hypoxic exercise performance. McCall and Frierson (1997) showed that only two genetic loci were principally responsible for dramatic differences in the duration of hypoxic treadmill exercise in mice following 8 weeks exposure to hypobaric hypoxia. Two inbred strains of mice, BALB/cByJ (C) and C57BL/6J (B6), were crossed to yield an F1 hybrid. The three strains had moderately different capacities for treadmill exercise under hypoxia (380 torr) if they were not previously exposed to hypoxia. However, following 8 weeks exposure to hypobaric hypoxia, the C mice showed no improvement in hypoxic exercise tolerance, B6 mice showed considerable improvement, while F1 mice showed a dramatically improved capacity for hypoxic exercise. The present study was designed to test the hypothesis that either strain-specific differences in skeletal muscle or hypoxia-induced remodeling of skeletal muscle was at least partially responsible for the large differences in heritable hypoxic endurance capacity. Specifically, we predicted that a superior hypoxic exercise tolerance would be associated with a relatively high slow-twitch fiber composition, CD, and oxidative potential, similar to the profile observed in muscle following endurance training.

2. Materials and methods

2.1. Animals and hypoxic exposure

The animals and treatments were identical to that described in McCall and Frierson (1997), who demonstrated the heritability of hypoxic exercise tolerance (t_{et}) among three strains of mice (t_{et} is the duration of hypoxic treadmill exercise on a 15° incline at 40 cm s⁻¹ under 1/2 atm PO₂). Two inbred strains of mice, C57BL/6J (B6) and BALB/cByJ (C), obtained from The Jackson Laboratory, were crossed to yield a hybrid (F1). Mice were housed in polycarbonate cages under a laminar flow hood at 23 ± 1 °C on a 12:12 h light–dark cycle. They were provided Agway 3000 Mouse Chow and water ad libitum. When mice were 8 weeks old, they were transferred from the colony to a two-compartment Vacu-dyne environmental chamber. Mice were exposed to either hypobaric hypoxia equivalent to an altitude of 5500 m (1/2 atmospheric pressure) or normobaric normoxia for an additional 8 weeks.

2.2. Muscle preparation

At 16 weeks of age, mice were weighed and euthanized with 100% CO₂. The extensor digitorum longus (EDL), a predominantly fast-twitch muscle, and the soleus (SOL), a predominantly slow-twitch muscle, were isolated and the *in vivo* resting length was measured while the knee and ankle joints were in full flexion. The muscles were dissected while being flushed with saline equilibrated with a 95/5% O₂/CO₂ mixture (pH 7.4). The tendons of each muscle were tied off with 5–0 surgical silk and the muscles were secured to a small frame at resting length. Muscles to be used for histochemistry were frozen in isopentane cooled in liquid N₂ and then embedded in Gum Tragacanth (ICN Biochemicals). Muscles to be used for electrophoresis or enzyme assays were freeze-clamped in liquid N₂. All samples were stored at –85 °C until use.

2.3. Histochemistry

At the midpoint of each muscle, serial transverse sections (10 µm thick) were cut at –19 °C on a Reichert–Jung Cryocut 1800 cryostat. Sections were pre-incubated in either acid or alkali medium prior to staining for myosin–ATPase activity (Padykula and Herman, 1955). Acid pre-incubations were carried out for 5 min in 200 mM barbital acetate buffer with the pH adjusted to either 4.0 (Sillau and Bancho, 1977) for capillaries, or 4.3 and 4.6 for muscle fiber type composition (Brooke and Kaiser, 1970). Alkali pre-incubations were performed with slight modifications to the method described by Guth and Samaha (1969). Sections were fixed for 2 min in buffered formaldehyde (8% formaldehyde, 200 mM cacodylate, 68 mM CaCl₂,

and 340 mM sucrose, pH 7.6), followed by a 2 min rinse in 100 mM Tris–HCl containing 18 mM CaCl₂ (pH 7.8). Sections were pre-incubated for 15 min with the pH adjusted to 10.4 in 18 mM CaCl₂ and Sigma 221 buffer. All sections were incubated at 37 °C for 20 min in incubation medium (100 mM sodium barbital, 18 mM Ca²⁺ and 12 mM ATP, pH 9.4) and then stained for myosin-ATPase activity (Padykula and Herman, 1955). Slides were viewed on an Olympus BH-2 light microscope and digital images collected with a Diagnostic Instruments SPOT Camera.

Muscle fiber type abundance, fiber-type cross-sectional area (FCSA), CD, capillary/fiber (C/F) ratios, and capillaries around a fiber (CAF) were analyzed using a combination of stereological (Howard and Reed, 1998) and image analysis methods (Image Pro Plus Software version 4.5). A square lattice counting grid with an area of 0.1 mm² and 49 points was placed over an image of the muscle in four randomly selected, non-overlapping regions. The area associated with each point on the grid was 0.002 mm². In each grid the total number of each fiber type was counted in order to calculate the numerical fiber type composition. Two hundred fibers were typically counted in each EDL and SOL. The percentage of the area covered by each fiber type was estimated by point counting within each grid (excluding extracellular space). The fiber cross-sectional area of each fiber type was estimated by dividing the fraction of the area covered by each fiber type by the number of fibers of that type.

Sections pre-incubated in acidic medium (pH 4.0) yielded darkly stained capillaries that had sufficient contrast from the rest of the section that the software's threshold function could be used to count capillaries. CD was defined using muscle fiber cross-sectional area as a reference space, and was calculated by dividing the number of capillaries by the muscle fiber cross-sectional area sampled. The total number of capillaries was then divided by the total number of fibers contained in the same regions to give a capillary-to-fiber (C/F) ratio. Capillaries around a fiber (CAF) was calculated by manually counting the number of capillaries around 20 randomly selected type 1 (CAF 1) and type 2 fibers (CAF 2) in each muscle. Only vessels with a diameter < 10 μm were counted, which will largely be comprised of capillaries but may also include terminal arterioles or venules.

2.4. Electrophoresis

MHC isoform composition was analyzed by SDS–PAGE using a modification of the method described in Talmadge and Roy (1993). Frozen tissues were homogenized in nine volumes of buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris, pH 6.8). The homogenate was centrifuged for 30 min at 13,800 × *g*. The pellet was resuspended in an equal volume of saline solution (60 mM KCl, 30 mM imidazole, 2 mM MgCl₂,

pH 7.0) and centrifuged at 750 × *g*. This two step process was repeated a total of three times to obtain washed myofibrils (Solaro et al., 1971). The pellet was then re-suspended in an equal volume of suspension solution (150 mM KCl, 20 mM Tris, pH 7.4). The samples were boiled in sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) for 2 min at a final concentration of approximately 0.175 mg/ml (as determined by Bradford protein assay). Samples were stored in five volumes of 60% glycerol, 40 mM Na₄P₂O₇, 2 mM EGTA, 1 mM 2-mercaptoethanol at pH 8.8.

The separating gel was composed of 30% glycerol, 8% acrylamide:bis-acrylamide (50:1), 200 mM Tris (pH 8.8), 100 mM glycine, and 0.4% SDS (w/v). The stacking gel was composed of 30% glycerol, 4% acrylamide:bis-acrylamide (50:1), 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% SDS. Gels were run on a Hoefer SE250 minigel apparatus. The upper buffer contained 100 mM Tris (base), 150 mM glycine, 0.1% SDS, and 800 μl 2-mercaptoethanol. The lower buffer contained 50 mM Tris (base), 75 mM glycine, and 0.05% SDS. Gels were run for 30 h at 70 V in a cold-room maintained at 4 °C, silver stained (Bio-Rad) and imaged using a Bio-Rad Gel Doc 2000 system. Optical density was calculated using Quantity One (Bio-Rad) software.

2.5. Citrate synthase activity

Flash frozen tissue was placed in ice-cold buffer containing 50 mM Tris (pH 7.3), 1 mM EDTA, 2 mM MgCl₂, and 2 mM DTT, homogenized for 30 s using a Fisher Power Gen 125 sawtooth homogenizer, and sonicated for 5 s using a Fisher 60 sonic dismembrator. The homogenate was centrifuged at 4 °C for 20 min at 12,000 × *g* and the supernatant was frozen at –85 °C until use. Citrate synthase (CS) activity was measured using a Pharmacia Ultrospec 4000 spectrophotometer at 25 °C as described previously (Boyle et al., 2003).

2.6. Statistical analysis

All data are presented as means ± SEM. Using JMP statistical analysis software version 4.0 (SAS Institute, Cary, NC, USA) a 2-way analysis of variance (ANOVA) was used to analyze the effects of hypoxia, mouse strain, or an interaction of the two on muscle fiber and MHC composition, CD and CS activity. When appropriate, differences between groups were tested with Tukey's HSD test. A prospective power analysis of initial data indicated that five replicates (animals) per group were sufficient to detect a 20% change with a power >0.9 for all variables. Therefore, an *n* ≥ 5 was utilized for each group in all experiments. A retrospective power analysis carried out on our final data sets following two-way ANOVA indicated that the actual power of our analyses was often much greater than this. We

Table 1

Body mass of animals used in present study and previously measured hypoxic exercise tolerance (t_{et}) from McCall and Frierson (1997)

	C		B6		F1	
	N	H	N	H	N	H
<i>n</i>	22	21	22	20	28	34
Body mass (g)	26.48 ± 0.88	21.63 ± 0.71*	23.92 ± 0.85	23.11 ± 0.64	29.44 ± 1.08†	23.12 ± 0.49†*
t_{et} (min)	9.5 ± 1.0	12.02 ± 1.8	21.38 ± 1.1	42.34 ± 1.2*	17.16 ± 1.2	77.62 ± 2.8*
range (min)	2.5–25.5	2.5–31.8	10.8–33.7	28.3–69.0	5.9–36.3	52.2–112.1

A significant treatment effect was detected for body mass, and a * indicates a significant difference within a strain between normoxia and hypoxia exposure. Where a significant strain effect was detected, a † indicates that the strain was significantly different from the other two. N refers to normoxic treatment and H refers to hypoxic treatment.

were able to detect effects of strain, treatment or an interaction of the two that resulted from a 10% change with a power >0.9 for 40% of our tests. An effect resulting from a 20% change could be detected with a power of 0.9 for 90% of our measurements. The only results with a lower power were our measurements of fractional area and numerical composition for very rare fiber types (type 2x in SOL and type 1 in EDL), which make up \approx 1% of the fiber area (see Section 3).

3. Results

3.1. Body mass

Table 1 shows body mass of mice used in the present study, as well as the previously determined hypoxic treadmill exercise time, t_{et} (McCall and Frierson, 1997). Two-way ANOVA detected significant effects of treatment ($F=36.1$, $P<0.0001$), strain ($F=7.24$, $P=0.001$) and an

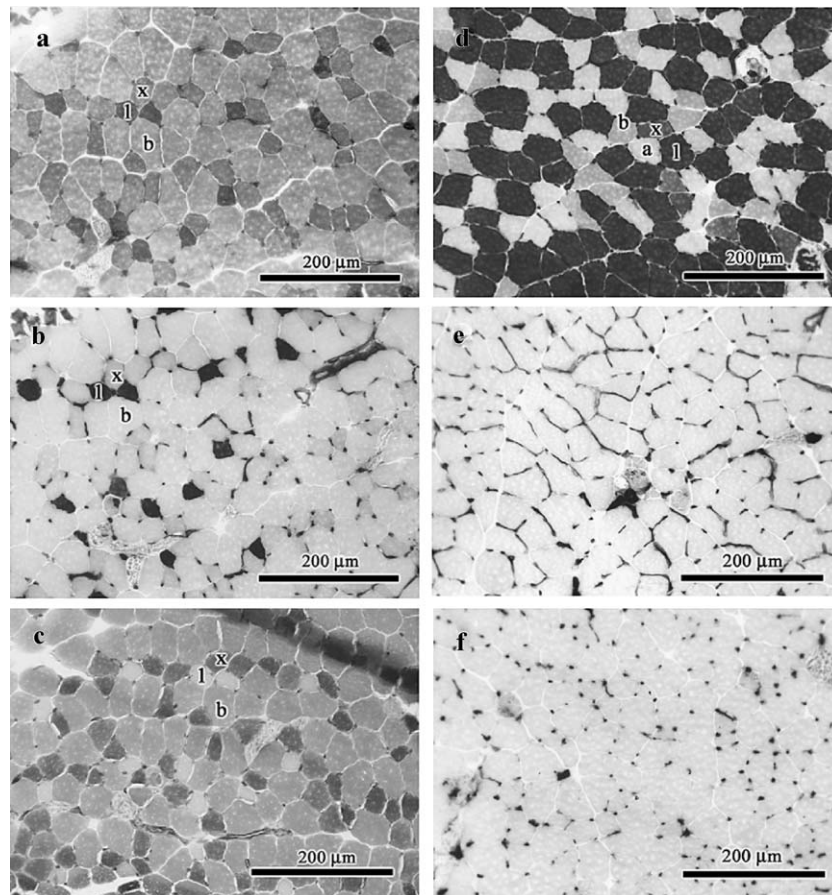


Fig. 1. Examples of transverse sections of muscle from F1 mice with histochemical staining for myosin ATPase preincubated at (a) pH 4.6 in EDL, (b) pH 4.3 in EDL, (c) pH 10.4 in EDL, (d) pH 4.6 in SOL, (e) pH 4.0 in SOL, (f) pH 4.0 in EDL. Fiber types I, 2a, 2x and 2b were identified in the SOL and fiber types I, 2x and 2b were identified in the EDL. Serial sections in (a–c) show staining patterns of fiber types at different preincubations in the EDL. The section in (d) demonstrates that all 4 fiber types in SOL could often be resolved at a single pH. The examples in (e) and (f) show the difference in capillarity between the two muscles.

interaction of strain and treatment ($F=6.2$, $P<0.003$) on body mass (Table 1). Hypoxia exposed mice of all strains had a lower body mass than those under normoxia. Unpublished results have shown that the three strains lose body mass during the first week of hypoxia exposure, but in subsequent weeks mass increases at a rate comparable to that seen in mice acclimated to normoxia, although they do not recover to levels equal to normoxia exposed mice by the time of sacrifice.

3.2. Fiber type composition

The staining patterns of the fiber types are shown for the SOL and EDL (Fig. 1a–d). The fractional area of the SOL was dominated by fiber types 1 and 2a, with a small contribution from types 2b and 2x (Fig. 2a). Two-way ANOVA found no significant strain effects in the fractional area of each fiber type. However, a significant treatment effect was detected for type 2b ($F=6.72$, $P<0.05$), which increased significantly following hypoxic exposure in C and F1 mice, and type 2a ($F=4.28$, $P<0.05$), which decreased significantly following hypoxic exposure in B6 mice (Fig. 2a). The fractional area of the EDL was almost exclusively comprised of types 2b and

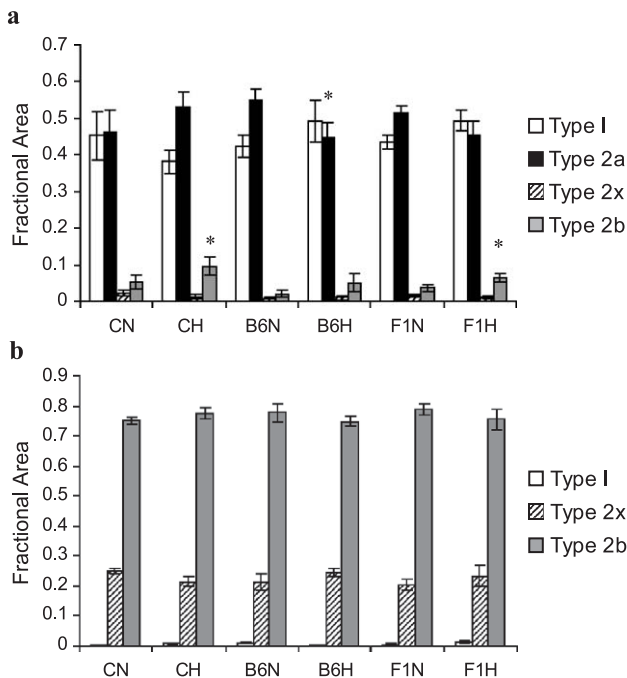


Fig. 2. Fractional area of each fiber type in the (a) SOL and (b) EDL. In the SOL, n values were CN=5, CH=6, B6N=6, B6H=5, F1N=6 and F1H=5. In the EDL, n values were CN=7, CH=6, B6N=7, B6H=7, F1N=6 and F1H=5. A significant treatment effect was detected for types 2a and 2b of the SOL, and the * indicates a significant change following hypoxic exposure. In the EDL, a significant interaction was detected in type 1 fibers, where type 1 fibers in F1H mice were significantly higher than B6H, CN, and CH treatment groups (not indicated on figure). N refers to normoxic treatment and H refers to hypoxic treatment.

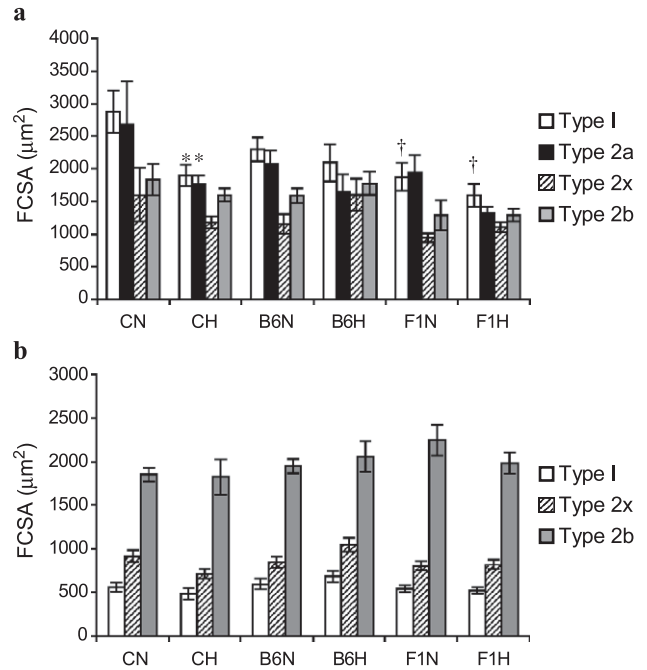


Fig. 3. Fiber cross-sectional area (FCSA) of each fiber type in the (a) SOL and (b) EDL. The n values are the same as in Fig. 2. A significant treatment effect was detected in types 1 and 2a of the SOL, and the * indicates a significant change following hypoxic exposure. Where a strain effect was detected, the † indicates type 1 fibers in F1 mice were significantly different from C and B6 mice. In the EDL, an interaction of strain and treatment was detected where B6 mice had a significant increase in type 2x FCSA following hypoxic exposure (not indicated on figure). In addition, type 2x FCSA in B6H mice was significantly higher than in CH mice (not indicated on figure).

2x with a slight contribution from type 1 fibers (Fig. 2b). There was a significant interaction of strain and treatment in the type 1 fiber fractional area ($F=3.71$, $P<0.05$), which was significantly higher in the EDL of the best performing hypoxic F1 animals than in normoxic C and F1 mice, and hypoxic B6 mice (Fig. 2b). The numerical composition data mirrored that for fractional area (data not shown).

A significant effect of treatment on FCSA was detected in the SOL. FCSA was significantly lower following hypoxic exposure in type 1 ($F=7.42$, $P<0.05$) and 2a fibers ($F=6.71$, $P<0.05$) of SOL in C mice (Fig. 3a). Also, a strain effect was detected ($F=5.05$, $P<0.05$), which resulted from a significantly lower type 1 FCSA in F1 mice than in B6 and C mice. An interaction effect was not significant, but the SOL of C mice showed a substantially larger decrease in the FCSA ($\sim 1000 \mu\text{m}^2$) of type 1 fibers than B6 ($\sim 150 \mu\text{m}^2$) or F1 mice ($\sim 270 \mu\text{m}^2$) following hypoxic exposure. A significant strain and treatment interaction was detected in the EDL ($F=4.7$, $P<0.05$). Type 2x FCSA increased significantly only in B6 mice following hypoxic exposure while no change occurred in the other strains (Fig. 3b). Type 2b fibers in the SOL and EDL of all three strains showed no significant change in FCSA after hypoxic exposure (Fig. 3).

Table 2
Capillary/fiber ratio (C/F), capillaries around a fiber (CAF), and capillary density (CD)

Muscle and mouse	Treatment	n	C/F ratio	CAF Type 1	CAF Type 2	CD (# / mm ²)
<i>SOL</i>						
C	N	6	2.34 ± 0.06	6.18 ± 0.28	5.79 ± 0.13 [†]	1368 ± 67
	H	5	2.56 ± 0.14	6.34 ± 0.21	5.59 ± 0.15 [†]	1178 ± 58
B6	N	6	2.06 ± 0.09	6.09 ± 0.15	5.35 ± 0.16	1260 ± 221
	H	6	2.33 ± 0.05	5.98 ± 0.19	5.41 ± 0.15	1326 ± 140
F1	N	6	2.31 ± 0.12	5.49 ± 0.20 [†]	5.38 ± 0.35 [†]	1206 ± 44
	H	5	2.19 ± 0.09	5.56 ± 0.16 [†]	5.10 ± 0.12 [†]	1273 ± 64
<i>EDL</i>						
C	N	8	1.56 ± 0.08	3.74 ± 0.13	3.89 ± 0.08	991 ± 53 [†]
	H	7	1.50 ± 0.06	3.68 ± 0.14	4.00 ± 0.07	1095 ± 53 [†]
B6	N	5	1.42 ± 0.14	3.04 ± 0.04	3.66 ± 0.11	874 ± 83
	H	6	1.30 ± 0.06	3.29 ± 0.28	3.79 ± 0.08	764 ± 83
F1	N	7	1.52 ± 0.06	3.46 ± 0.19	3.85 ± 0.11	878 ± 39
	H	5	1.46 ± 0.04	3.43 ± 0.12	3.80 ± 0.07	923 ± 64

Where a significant strain effect was detected, a † indicates the strain is significantly different from the other two, except for CAF Type 2 in SOL, where the † indicates that the C and F1 strains were significantly different from each other. N refers to normoxic treatment and H refers to hypoxic treatment.

3.3. Capillarity

Capillaries were clearly visible in sections that were preincubated at pH 4.0 prior to staining for myosin ATPase activity (Fig. 1e, f). In the SOL, capillaries were more abundant and more frequently sectioned longitudinally, due to a higher tortuosity, than in EDL (Fig. 1e, f). The values for C/F ratio, CAF, and CD for the SOL and EDL are presented in Table 2. No significant effect of chronic hypoxia on any of these parameters was detected for SOL or EDL. However, in the SOL, a significant strain effect was detected for CAF 1 ($F=6.03$, $P<0.01$) and CAF 2 ($F=5.12$, $P<0.05$). These effects resulted primarily from the significantly lower CAF 1 and CAF 2 in F1 mice than C and B6 mice. However, in the SOL the smaller FCSA in the F1 mice (Fig. 3a) appears to offset the lower CAF1, which may explain why there are no significant strain effects for

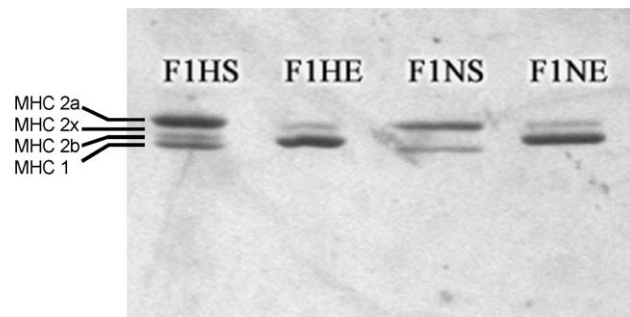


Fig. 4. Typical gel showing MHC isoforms in the SOL (S) and EDL (E) from F1 mice exposed to normoxia (N) or hypoxia (H). This gel illustrates the difference in band patterns between the SOL and EDL. In the SOL extracts MHC 2b bands were faint (this band is not visible in lane 3 due to a lower protein load). An MHC 2x band could not be quantified in the SOL because its low intensity required heavy protein loading to allow detection, and this led to overlap with the dominant MHC 2a band (as seen in lane 1). In the EDL (lanes 2 and 4), only MHC bands 2x and 2b were analyzed since MHC 1 bands were visible only when gels were overloaded, which led to biases in quantitation.

CD (Table 2). In the EDL, a strain effect ($F=6.95$, $P<0.01$) resulted from the significantly higher CD in C mice than in B6 and F1 mice.

3.4. Myosin heavy chain composition

The electrophoretic separation of the different MHC isoforms is shown in Fig. 4. Although four fiber types were

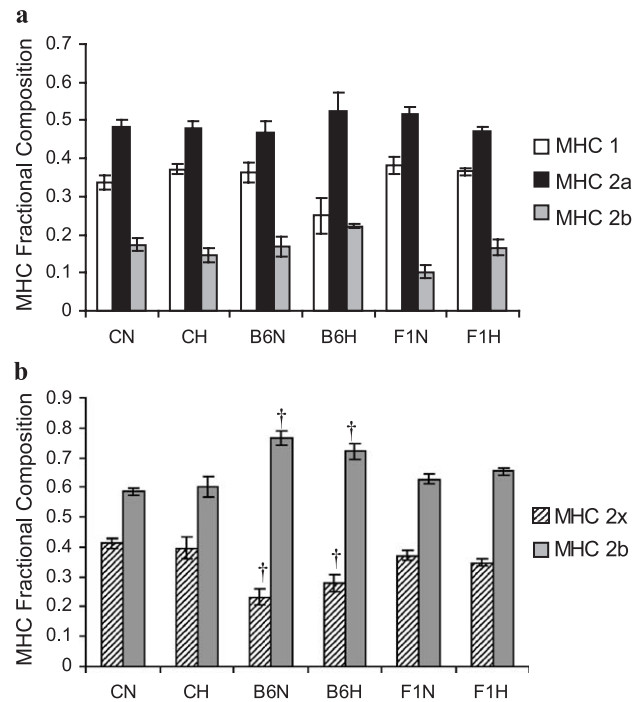


Fig. 5. MHC composition of a) SOL and b) EDL. In the SOL, n values were CN=5, CH=5, B6N=5, B6H=5, F1N=5 and F1H=8. In the EDL, n values were CN=6, CH=5, B6N=6, B6H=5, F1N=9 and F1H=11. A significant strain effect was detected in the MHC composition of the EDL, and the † indicates the MHC composition of B6 mice was significantly different from C and F1 mice.

found histochemically, only three bands (2a/2x, 2b, and I) were separable on gels due to overlap of the dominant MHC 2a band with the very faint 2x band. Difficulty separating MHC 2a and 2x in mouse SOL has been reported previously (Marechal et al., 1995; Zardini and Parry, 1994), and since type 2x fibers make up a very small fraction of the SOL (Fig. 2a; Termin et al., 1989) the MHC 2x isoform was not analyzed. In the EDL the low fractional composition of type 1 fibers (Fig. 2b) meant that MHC I could only be observed by overloading the gel with extract, which introduces quantification difficulties, so we analyzed only MHC 2x and 2b, which constitute >99% of the total.

In the SOL, the increase in 2b fiber composition following hypoxia (Fig. 2a) was also seen in the MHC 2b data for B6 and F1 mice, although the treatment effect was not significant ($F=3.15$, $P=0.08$) (Fig. 5a). Chronic hypoxia did not affect the MHC composition of the EDL, however, a significant strain effect was detected in MHC 2b ($F=23.68$, $P<0.0001$) and MHC 2x ($F=23.68$, $P<0.0001$). The B6 strain had a significantly higher MHC 2b (and therefore, significantly lower 2x) composition than did C and F1 mice (Fig. 5b). This strain effect was not observed for fiber type composition, and the fiber type and MHC data were not in complete agreement for C and F1 mice in EDL (Figs. 2b, 5b). This suggests that in EDL from C and F1 mice, some 2b fibers also contain a small fraction of the MHC 2x isoform, which is consistent with other reports of hybrid fibers in mouse skeletal muscle (Gorza, 1990; Hamalainen and Pette, 2000; Zahn et al., 1998; Zardini and Parry, 1994).

3.5. Citrate synthase activity

There were significant strain effects in CS activity for both the SOL ($F=5.39$, $P<0.05$), where the mean activity

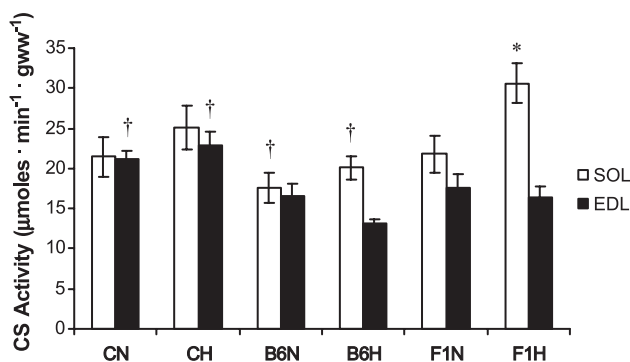


Fig. 6. CS activity in SOL (open bars) and EDL (filled bars). In the SOL, n values were CN=5, CH=5, B6N=5, B6H=5, F1N=5 and F1H=6. In the EDL, n values were CN=5, CH=5, B6N=5, B6H=6, F1N=5 and F1H=6. A significant strain effect was detected in both the SOL and the EDL, and the † indicates the strain that was significantly different from the other two strains. A treatment effect was detected in the SOL, and the * indicates that the CS activity in F1 SOL from hypoxia treated mice was significantly greater than that of normoxia treated mice. N refers to normoxic treatment and H refers to hypoxic treatment.

in B6 mice was significantly lower than that of the C or F1 mice, and for the EDL ($F=15.60$, $P<0.0001$), where mean activities of B6 and F1 mice were significantly lower than that of C mice (Fig. 6). There was also a significant treatment effect in the SOL ($F=7.12$, $P<0.05$), and pairwise comparisons revealed that the CS activity from hypoxic F1 mice was significantly greater than that from normoxic F1 mice (Fig. 6).

4. Discussion

The major finding of the present study was the remarkable consistency of skeletal muscle properties across both mouse strain and treatment. Many studies have examined the effects of chronic hypoxia on skeletal muscle fiber type composition, oxidative capacity and capillarity. This study differed in that the animals compared had striking differences in hypoxic exercise tolerance following exposure to hypoxia (McCall and Frierson, 1997; Table 1). Despite the genetically based performance differences; however, the results of the present study are in agreement with the bulk of the literature on mature mammals in that muscle fiber type, MHC composition and CD were largely unaffected by exposure to chronic hypoxia (Green et al., 1989; Hoppeler and Vogt, 2001; Ishihara et al., 1995; Jackson et al., 1987; Saltin et al., 1980; Sillau et al., 1980; Takahashi et al., 1992). More importantly, the few slight differences among strains and treatment that were observed did not follow a pattern that obviously related to hypoxic exercise tolerance. Therefore, we must reject our hypothesis and conclude that differences in these classical indicators of muscle remodeling do not contribute substantially to the differences in hypoxic exercise tolerance. The lack of change in muscle observed here is similar to the observations of Zahn et al. (1998). These authors found that mice that were artificially selected for 10 generations for high levels of voluntary wheel running did not have altered fiber-type composition or oxidative capacity in medial gastrocnemius. The results of Zahn et al. (1998) are of particular relevance to the present paper in that, in the absence of training, heritable differences in an endurance performance variable were not associated with substantial differences in fiber type or oxidative capacity.

While there was no clear association of fiber type composition and t_{et} , several points warrant further consideration. In the SOL, the increase in type 2b fractional area (Fig. 2a) and MHC 2b composition (Fig. 5a) following hypoxic exposure results in part from the reduction of FCFA in type 1 and 2a fibers (Fig. 3a). However, the increase in 2b numerical composition (data not shown) indicates some fiber transformation to type 2b as well. Increased 2b fiber composition following hypoxic exposure has also been seen in developing rat SOL, where the change in composition was actually a result of the inhibition of the substantial fiber shift from fast to slow fibers that occurs during the first

8 weeks of age (Ishihara et al., 1995; Sillau and Banchero, 1977; Wigston and English, 1992). This maturational fiber type shift in the rat is in contrast to the mouse SOL, which shows no significant change from fast to slow fiber types over the first year of life (Wigston and English, 1992). However, due to its relatively small contribution to SOL cross-sectional area ($\approx 5\%$), the small increase in 2b composition may not substantially alter whole muscle contractile and/or metabolic function.

Another interesting change in the SOL following hypoxic exposure was the reduction of FCSA for fiber types 1 and 2a in the C mice (Fig. 3a). These fiber types were significantly larger in the C mice than in the B6 or F1 mice following normoxia exposure, but after hypoxia exposure the C mice had type 1 and 2a FCSA values that were comparable to the other two strains. The reduction in FCSA under hypoxia may imply that these relatively large fibers have cellular diffusion distances that are too great to support adequate rates of oxygen flux under hypoxia. Also, a significant strain effect in the SOL resulted from the fact that F1 mice had a significantly lower FCSA of type 1 fibers than either of the two parental strains. It is possible that the smaller type 1 fibers in SOL contribute somewhat to the superior hypoxic exercise tolerance of this strain by virtue of the reduced oxygen diffusion distances, although this is likely offset by the relatively low CAF1 of these fibers (Table 2).

The fiber type composition of the EDL was even less variable among strains and treatment than in the SOL. The significantly higher type 1 fractional area of the EDL in the F1 hybrid following hypoxia is also probably not functionally meaningful in terms of hypoxic endurance exercise due to the very small fraction ($<1\%$) of the total muscle cross-sectional area represented by type 1 fibers (Fig. 2b). No other effects of hypoxia on fiber type composition or MHC composition in the EDL were seen; however, there were significant differences in MHC composition between mouse strains (Fig. 5b). It is interesting that in the EDL the worst-performing C mice had an MHC composition nearly identical to the best performing F1 mice, which is consistent with the notion that MHC composition does not correlate with hypoxic exercise tolerance in these strains.

Although the consistency of muscle fiber type and MHC isoform composition suggests that these muscle properties may not be associated with the differences among strains in hypoxic exercise tolerance, some of the unique qualities of mouse skeletal muscle should be noted. For instance, most mammals contain a lower proportion of fast-twitch fibers in the SOL than the mouse (Reichman and Pette, 1984), and the oxidative capacity of fast-twitch fibers in the mouse is higher than in other mammals as well (Pette and Staron, 1998). This suggests that fast muscles of the mouse are designed for more sustained activity, or for a rapid post-contractile recovery, and cannot be easily induced to attain a substantially higher oxidative potential (Pette and Staron, 1998). In support of this, Zahn et al. (1998) found that 2

months of access to a running wheel did not induce a change in MHC composition in mouse skeletal muscle, compared to sedentary controls. Further, Pette and Vrbova (1992) demonstrated that chronic low frequency stimulation easily induced fiber type transitions from fast to slow in rabbit, guinea pig, and rat but not in mouse muscle. Thus, the absence of fiber type differences among strains with dramatic differences in endurance capacity may reflect the fact that fiber type composition is a less malleable parameter in mouse than in other mammals.

The lack of a significant effect of chronic hypoxia on capillarity in the present study is in agreement with several studies on mammalian skeletal muscle (Banchero, 1987; Bigard et al., 1991; Mathieu-Costello, 1989; Poole and Mathieu-Costello, 1989, 1990; Sillau and Banchero, 1979; Sillau et al., 1980; Snyder et al., 1985). It is also interesting that the significant strain effects that were observed did not follow our expectations based on differences in hypoxia tolerance; the values for CAF in SOL and CD in EDL were highest in the worst performing C mice, although as noted above the relatively large CAF values in SOL were associated with larger fiber diameters in this strain. It is possible, however, that the relatively high capillarity in the C mice may relate to the fact that this strain has been shown to have a greater capacity for treadmill exercise under normoxic conditions than the B6 mice (Lightfoot et al., 2001). It is intriguing that the best performing parental strain under normoxia is the worst performer under hypoxia, and this may suggest an increased sensitivity to hypoxia in C mice. However, when examining the three strains together, the absence of positive correlations between capillarity and t_{et} suggests that this variable is not a principal mediator of hypoxic exercise tolerance.

CS activity differed among groups to a greater extent than the other measured variables, although the pattern of variation is difficult to interpret with respect to hypoxic exercise tolerance (Fig. 6). While CS is generally a good indicator of oxidative capacity due to its position as a key entry point in the citric acid cycle, the activity of this enzyme may not necessarily mirror that of other aerobic indicators, such as enzymes of the electron transport system. However, measurements of cytochrome *c* oxidase (COX) activity in EDL and SOL in the same strain/treatment groups used in the present study correlate fairly well with our CS activity measurements, although the COX activity was less variable than CS activity (Ernst, 2003). In both the SOL and EDL, the C mice, which performed the worst on the treadmill, had relatively high CS activities while the B6 mice, which were intermediate performers, had relatively low CS activities, which is opposite to our predictions. Again, this may relate more to the higher endurance capacity of C mice than B6 mice under normoxic conditions (Lightfoot et al., 2001), rather than during hypoxic exercise. It is interesting to note, however, that the highest activities were found in the SOL of the best performing hypoxic F1 mice. This is consistent with our hypothesis that a relatively

high oxidative capacity is associated with an enhanced capacity for hypoxic treadmill exercise. The relatively large range of CS activities, compared to other measured variables, may also indicate that metabolic characteristics of these muscles may be more plastic than fiber-type composition or capillarity.

Considering the relatively low variability among the measured properties of skeletal muscle in the present study, how might we explain the large differences in hypoxic exercise tolerance among strains? First we must consider the possibility that subtle changes that we could not detect (i.e. <10% change) may be important determinants of t_{et} . This is particularly important when we consider our measurements in the context of the concept of symmorphosis (Taylor and Weibel, 1981). The co-adjustment of multiple parameters that are part of either a serially linked pathway, such as oxygen delivery to the tissues, or a branched network, such as the pathways associated with metabolic substrate utilization/delivery, may involve only slight changes to individual components (Taylor et al., 1987; Weibel et al., 1996). Our limited data, however, suggest that this is not the case, since we found relatively high capillarity and CS activities in the worst-performing C mice. This indicates that these structural components of the oxygen delivery and substrate utilization pathways are not principal contributors to the differences in t_{et} . This of course does not preclude the possibility that other aspects of the oxygen supply pathway are important. Rats that have been artificially selected for high endurance capacity have an elevated $\dot{V}O_2\max$ that is principally the result of an increased capacity for oxygen extraction from the capillaries by the tissues, although the structural basis for this difference is unknown (Henderson et al., 2002). Henderson et al. (2002) have found that this relationship holds under both normoxic and hypoxic exercise. It has also been shown in an extensive series of studies that endurance capacity is associated with enhanced intracellular stores of glycogen and fatty acids (Weibel et al., 1996), and an increased reliance on glucose oxidation under hypoxia has long been postulated to be advantageous because of the higher ATP yield per O_2 consumed compared to fatty acid oxidation (e.g. Hochachka et al., 1998). There also may be an increased capacity for anaerobic glycolysis to facilitate hypoxic exercise (Vogt et al., 2001), which may require adjustments in related properties such as an enhanced intracellular pH buffering capacity and elevated rates of lactate transport and clearance. Further, it has been suggested that acclimation/adaptation to hypoxia induces a tighter coupling of metabolic ATP production and ATP consumption processes in muscle fibers, leading to a reduced perturbation of the intracellular adenylate pool and lower lactate accumulation during exercise (Hochachka et al., 2002).

Thus, as yet unmeasured adjustments in cardiovascular properties and in the components of energy metabolism and their control in muscle may be more important to t_{et} than the changes in the structural parameters measured in the present

study. However, it should be pointed out that a unifying set of responses that explains the differences in t_{et} in our model may not exist. In examining a whole animal phenotypic character that is responsive to many adjustable physiological structures, it is very likely that the magnitude or even the direction of responses at some levels will differ among the strains. In an integrated system, this will be associated with co-adjustment of other structures that may lead unexpected variation among the strains. The lack of substantial differences among strains or treatment-induced changes in the present study, however, suggests that the measured properties that are proposed to be part of the ‘hypoxia tolerance/endurance exercise phenotype’ in skeletal muscle, as described by Hochachka et al. (1998, 1999), are not among the inherited physiological attributes that promote hypoxic performance in mice.

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