Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia

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Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia. Am J Physiol Heart Circ Physiol 281: H241–H252, 2001.—Whether chronic hypoxia causes angiogenesis in skeletal muscle is controversial. Male Wistar rats, 5–6 wk of age, were kept at constant 12% O₂ for 3 wk, and frozen sections of their postural soleus (SOL), phasic extensor digitorum longus (EDL), and tibialis anterior (TA) muscles were compared with those of normoxic controls. Capillary supply increased in SOL muscles (capillary-to-fiber ratio (C/F) = 2.55 ± 0.09 hypoxia vs. 2.17 ± 0.06 normoxia; capillary density (CD) = 942 ± 14 hypoxia vs. 832 ± 20 mm⁻² normoxia, P < 0.01) but not in EDL muscles (C/F = 1.44 ± 0.04 hypoxia vs. 1.42 ± 0.04 normoxia; CD = 876 ± 52 hypoxia vs. 896 ± 24 mm⁻² normoxia). The predominantly glycolytic cortex of TA muscles showed higher C/F after hypoxia (1.79 ± 0.09 vs. 1.53 ± 0.05 normoxia, P < 0.05), whereas the mainly oxidative TA core with smaller fibers showed no change in capillarity. The region of the SOL muscle with large-sized (mean fiber area 2,843 ± 128 μm²) oxidative fibers (90% type I) had a higher C/F (by 30%) and CD (by 25%), whereas there was no angiogenesis in the region with sparse (76%) and smaller-sized (2,200 ± 85 μm²) type I fibers. Thus systemic hypoxia differentially induces angiogenesis between and within hindlimb skeletal muscles, with fiber size contributing either directly (via a metabolic stimulus) or indirectly (via a mechanical stimulus) to the process.

capillary growth; histochemistry; hypoxemia; oxygen transport; rat

IT IS GENERALLY ACCEPTED that chronic systemic hypoxia, whether at high altitude or imposed experimentally by a hypoxic or hypobaric chamber, induces physiological adaptations that help to attenuate the impaired O₂ transport to tissue. These include an increase in minute ventilation and in hematocrit such that the O₂ content of the arterial blood is increased compared with nonadapted animals at reduced O₂ tensions (4, 47). Indeed, in rats exposed to 12% O₂ in a hypoxic chamber for 3–4 wk, the arterial O₂ content, blood pressure, and muscle blood flow underwent compensatory changes such that the gross O₂ delivery to skeletal muscle was equal to that of control rats breathing air (28). In the cerebral vasculature, chronic hypoxia induces structural changes including growth of new capillaries, which appear to be dilated, longer, and more tortuous than normal. The increase in capillary density (CD) therefore leads to reduced distances for diffusion of O₂ to the tissue parenchyma (25). Whether or not similar structural changes occur in the skeletal muscle vasculature remains controversial.

Earlier studies have suggested that exposure to high altitude is associated with an increase in skeletal muscle capillarity. Valdiva (48) reported that leg muscles of Andean guinea pigs born and reared at high altitude had a higher CD and capillary-to-fiber ratio (C/F), with a similar number of muscle fibers per unit area, than those of weight-matched guinea pigs reared at sea level. Cassin et al. (9) also reported a higher CD in the gracilis and plantaris muscle of rats exposed to high altitude (6,150 m; equivalent to 10% O₂) for 5 wk than in control rats at sea level. Similarly, Banchero et al. (5) found that dogs kept in a hypobaric chamber at a barometric pressure of 435 mmHg (equivalent to ~12% O₂) for 3 wk showed a twofold increase in CD in the sternothyroid muscle. Furthermore, Oelz et al. (31) reported a significantly higher CD and C/F in high-altitude (human) climbers. However, Banchero (4) maintained that such findings had been misinterpreted. The histological techniques of Valdiva (48) caused shrinkage of the tissues, making calculations from the data unreliable, and guinea pigs reared at high altitude were subjected to the double stress of cold plus hypoxia. Also, the results of Cassin et al. (9) could be explained by the substantially lower body mass of chronically hypoxic (CH) rats compared with controls, which produced a smaller fiber cross-sectional area (FCSA), suggesting no real increase in the number of capillaries. In Banchero et al.’s study (5), the increase in CD of the sternothyroid muscle could also be attributed to atrophy: a 30% reduction in FCSA secondary to a tracheostomy. Indeed, having shown that an increase in body mass associated with maturation has important effects on FCSA, C/F, and CD and that chronic hypoxia reduces weight gain or causes weight loss, Sillau and Banchero (40) compared CH rats ex-

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posed to 12.5% O₂ for 6 wk with weight-matched controls kept at sea level. They reported that FCSA and C/F were similar in soleus (SOL), gastrocnemius, and tibialis anterior (TA) muscles of hypoxic rats and weight-matched controls, and that the relationship between CD and body weight was not significantly different between groups. Similar findings were made in guinea pigs exposed to hypoxia (12.5% O₂) for 2–14 wk and control animals of a wide range of body weights (39). More recently, Poole and Mathieu-Costello (34) reported no change in capillarization of muscle in CH rats in which fiber size did not change, whereas Abdelmalki et al. (1) and Bigard et al. (6), who exposed rats to 13% or 12.5% O₂ for 9 or 12 wk, respectively, also concluded that any differences between muscle capillarity in control and CH rats could be attributed to differences in body mass. Finally, Hansen-Smith et al. (19) showed that enzymatic labeling of capillaries may be altered by hypoxia and that a nonenzymatic marker revealed no change in skeletal muscle capillarity in mice exposed to hypoxia for up to 3 wk.

However, a number of studies suggest that the issue deserves further investigation. For example, Snyder et al. (43) found that goslings exposed to hypoxia as embryos had a substantially increased C/F in gastrocnemius muscle with no change in FCSA, whereas rats that were exposed to 10% O₂ for 5 wk showed no change in C/F in the diaphragm or gastrocnemius muscle compared with controls of similar body mass but an increase in CD in the diaphragm that was associated with a decrease in FCSA. This raises the possibilities that chronic hypoxia as a cause for growth of new capillaries may be restricted to the early stages of maturation and that, in more mature animals, a shortening of intramuscular O₂ diffusion distances in active muscles may occur by a general reduction in FCSA or a selective loss of large fibers. On the other hand, high-altitude hypoxia in human subjects increased the activity of oxidative enzymes (44). In contrast, CH rats had an increased proportion of fibers with low oxidative enzyme activity in gastrocnemius and TA muscles (39) and an inhibition in the normal shift from type IIa (fast oxidative glycolytic) to type I (slow oxidative) fibers, which occurs during postnatal development in the SOL muscle (23, 24). Chronic hypoxia may therefore modify the capillary bed of skeletal muscle according to changes in muscle fiber type (22). Finally, there is recent evidence that within 7–21 days of chronic hypoxia (12% O₂), remodeling of the arteriolar tree occurs in skeletal muscle of the rat: the density of the arteriolar network is greatly increased by an increase in the number of arteriolar branches (41), which are mainly formed by arteriolarization of capillaries (35). Clearly, if new capillaries did not grow to match the increase in arteriolar density, the altered hemodynamics would result in less efficient peripheral O₂ exchange.

Given the uncertainties in the literature, the main aim of the present study was to determine whether or not chronic hypoxia per se induces capillary angiogenesis in skeletal muscle. Our investigations were made on CH rats and on normoxic controls of similar weight. Initially, we compared muscles that are routinely or tonically active (diaphragm and SOL) with those that are relatively inactive in a sedentary animal [extensor digitorum longus (EDL) and TA]. Our results indicated that chronic hypoxia can indeed induce capillary growth. Determining whether or not angiogenesis was dependent on FCSA and or muscle fiber type required further analyses on muscles that are very different with respect to these variables (SOL, EDL, and TA). We used an unbiased sampling protocol that, to our knowledge, has not been used in previous studies in this field and greatly reduces the data variance caused by inhomogeneities across the muscle section. Preliminary data have been reported in abstract form (11, 13).

MATERIALS AND METHODS
Experimental protocol. Experiments were performed on six male Wistar rats, 178 ± 6 g initial body wt (means ± SE), that were kept in a normobaric hypoxia chamber at 12% O₂ for 3 wk (CH rats) and on five weight-matched controls (normoxic rats). The body masses of these animals at the time of experimentation were 325 ± 5 g (means ± SE) and 326 ± 9 g, respectively (not significant [NS]). Animals were killed by an overdose of intraperitoneal pentobarbitone sodium, and the SOL, EDL, TA, and diaphragm muscles were then carefully separated from the surrounding tissue and weighed. A transverse section (~9 mm thick) was cut from the midbelly of each leg muscle, embedded in an inert mounting medium (Tissue-Tek, optimum cutting tissue compound), and snap-frozen in isopentane (2-methylbutane) cooled in liquid N₂ (~190°C). Samples of the diaphragm, each taken from the left anterior costal region, were treated in a similar fashion. Several sections of each muscle were cut (10-μm thick at ~22°C) and stained for alkaline phosphatase activity to identify all capillaries and for succinic dehydrogenase (SDH) and myosin-ATPase (preincubated at pH 4.55) to distinguish the three main muscle fiber types (14). In the stains for ATPase activity, dark staining indicates type I (slow oxidative), light staining indicates type IIA (fast oxidative glycolytic), and intermediate staining intensity indicates type IIB (fast glycolytic) fiber types; in the SOL muscle, pale staining indicates type IIC fibers. SDH activity was used as a check for the relative oxidative capacity (Fig. 1).

Analytical procedures. A square lattice counting frame (area 0.194 mm²) was placed over the image of a muscle section by use of a microscope drawing arm for a total magnification of ×250. Within each muscle section, all analyses were always performed on several different areas that were each in the same position relative to the long axis of the section and the section boundaries, e.g., starting three to four fibers from the edge and equidistant between sample fields (15) (see Fig. 2). Location was especially important in the outer cortex of the TA muscle, where fiber composition was very sensitive to the sample position. Within each circumscribed region, the relative muscle cross-sectional area occupied by each of the three main fiber types was quantified by a stereological point-counting method (15). An unbiased counting rule was used to estimate the CD (in number of capillaries/mm²), the C/F (in number of capillaries/total number of fibers), numerical fiber type composition of each fiber type (the total number of fibers in a sample/the appropriate number of fibers), and the average FCSA of each fiber type (fraction of the sample area occupied by each fiber type in μm²/the appropriate number of fibers) (15).

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Mathematical modeling. The influence of fiber size on intracellular O₂ tension of muscle fibers in relatively sedentary animals was explored by means of a model of peripheral oxygen supply based on a modified Krogh integration, utilizing in vivo values of resting arterial and venous PO₂ (26). This approach was compared with an analysis of the combined effect that changes in composition and fiber size have on muscle fiber PO₂ by means of a mathematical model of diffusion (20). Briefly, the maximum potential O₂ delivery (determined by the measured capillary supply) is balanced by O₂ consumption (scaled according to published data for mitochondrial volume), allowing for the diffusion distances involved (which varies with fiber size measured in this study) and O₂ permeability (from reference sources).

Statistical analyses. Unless stated otherwise, all data are presented as means ± SE. The values calculated for each region analyzed in each muscle were averaged to give a single value for each muscle. In addition, each of the regions within the SOL and EDL muscles were analyzed separately, whereas in the TA muscle the three regions in the cortex or core were grouped for analysis. Comparisons between muscles of normoxic and CH rats were made by factorial ANOVA and Fisher’s least significant difference post hoc analysis, with P < 0.05 considered significant.

RESULTS

Body and tissue mass. The body weights of the normoxic and CH rats were very similar (Table 1), although heart weight, particularly of the right ventricle, was significantly greater in CH rats than normoxic rats. As expected in animals with right heart hypertrophy, the lungs were also substantially heavier in CH than normoxic rats when expressed as either wet or dry weight (9 and 13%, respectively). The wet weight of the SOL, TA, and EDL muscles were significantly greater in CH than normoxic rats (Table 1). Dry weight of the TA muscle was also greater in CH than normoxic rats, and a similar trend was present for the SOL muscle (P = 0.06), but dry weight of the EDL muscle was similar in both groups of animals (Table 1).

Average muscle composition. Table 2 shows the data from all regions combined to give average values for each muscle. As noted previously (14), the fiber compositions of these muscles in normoxic rats were very different. The SOL muscle contained mainly type I fibers, with small proportions of type IIa and IIc fibers, whereas the EDL and TA muscles were predominantly comprised of type IIb fibers (70 and 65%, respectively), with smaller proportions of type IIa and I. When expressed in average values for whole muscles, chronic hypoxia had no significant effect on the proportions of any fiber type in any of the muscles (Table 2). The fiber composition of the diaphragm was not analyzed in the present study, but Egginton (14) showed it to be com-
posed of similar proportions of type I, IIa, and IIb fibers (31.5 ± 1.9, 39.2 ± 2.3, and 29.3 ± 2.6%, respectively). Furthermore, the average FCSA of the SOL muscle (2,403 ± 169 μm²) was substantially larger than that of the diaphragm or EDL muscle (∼1,500 μm²), being composed of the large type I fibers (see below), but there was no significant difference in the average FCSA between normoxic and CH rats for any muscle (Fig. 3). However, overt angiogenesis was evident in CH relative to normoxic rats, as reflected by a more extensive anatomical capillary supply (greater mean C/F) and an improved functional capillary supply (mean CD) in the diaphragm (by 17 and 16%, respectively) and SOL muscle (by 18 and 13%, respectively) but not in the EDL or TA muscles (Fig. 3).

The diaphragm had less distinct regions than the other muscles examined, with a similar composition and fiber size among all sample sites (Fig. 4). Type IIb fibers were the largest, being similar in size to those found in the TA cortex and SOL region 1, and were evenly distributed throughout the muscle. Angiogenesis occurred in all regions of the muscle, as shown by the C/F values, although modest differences in FCSA ensured that the increase in CD only reached statistical significance in region 2.

**Table 1. Body and tissue weights of normoxic and CH rats**

<table>
<thead>
<tr>
<th></th>
<th>Normoxic Rats</th>
<th>CH Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>312 ± 4.22</td>
<td>316 ± 4.09</td>
</tr>
<tr>
<td>Heart weight</td>
<td>995 ± 15.4</td>
<td>1,202 ± 52.5‡</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>646 ± 13.4</td>
<td>683 ± 32.1</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>237 ± 7.5</td>
<td>323 ± 30.5‡</td>
</tr>
<tr>
<td>Lung weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet weight</td>
<td>1,332 ± 48</td>
<td>1,588 ± 34‡</td>
</tr>
<tr>
<td>Dry weight</td>
<td>299 ± 12</td>
<td>339 ± 9*</td>
</tr>
<tr>
<td>EDL muscle weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet weight</td>
<td>134 ± 2.6</td>
<td>151 ± 4.7‡</td>
</tr>
<tr>
<td>Dry weight</td>
<td>31.1 ± 1.2</td>
<td>32.8 ± 0.5</td>
</tr>
<tr>
<td>SOL muscle weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet weight</td>
<td>139 ± 3.2</td>
<td>151 ± 3.5†</td>
</tr>
<tr>
<td>Dry weight</td>
<td>31.2 ± 1.6</td>
<td>34.6 ± 0.9</td>
</tr>
<tr>
<td>TA muscle weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet weight</td>
<td>561 ± 10.7</td>
<td>636 ± 13.9‡</td>
</tr>
<tr>
<td>Dry weight</td>
<td>134 ± 5.2</td>
<td>146 ± 2.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Body and tissue weights were measured in grams and milligrams, respectively. CH rats, chronically hypoxic rats; EDL, extensor digitorum longus; TA, tibialis anterior; SOL, soleus. *P < 0.05, †P < 0.01, and ‡P < 0.001 vs. normoxic rats.

**Table 2. Fiber composition (numerical density) in normoxic and CH rats**

<table>
<thead>
<tr>
<th>Muscles</th>
<th>TA</th>
<th>EDL</th>
<th>SOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>4.0±1.0</td>
<td>6.7±0.4</td>
<td>80.1±1.9</td>
</tr>
<tr>
<td>CH</td>
<td>4.9±0.9</td>
<td>6.6±0.6</td>
<td>76.8±2.5</td>
</tr>
<tr>
<td>Type IIa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>31.0±2.3</td>
<td>23.3±1.2</td>
<td>14.7±1.6</td>
</tr>
<tr>
<td>CH</td>
<td>31.2±1.7</td>
<td>26.3±1.2</td>
<td>19.4±2.2</td>
</tr>
<tr>
<td>Type IIb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>65.0±1.1</td>
<td>70.0±1.4</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>64.0±1.5</td>
<td>67.1±1.4</td>
<td></td>
</tr>
<tr>
<td>Type IIc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>5.2±1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>4.4±0.8</td>
<td></td>
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</tbody>
</table>

Values are means ± SE. Fiber composition was measured as numerical density (in %).
rats than they were in other regions, and there was a greater C/F in CH relative to normoxic rats (Fig. 6).

Within the SOL muscle, there was no significant difference in average size of type I or IIc fibers (2,483 ± 51 and 1,852 ± 60 μm², respectively), but type IIa fibers were larger in CH rats (2,300 ± 80 vs. 1,917 ± 108 μm² in controls, P < 0.01). C/F was greater in CH rats than in normoxic rats in regions 1 and 2 but not in region 3, and CD followed this trend with 25, 11, and 5% higher values in muscles of CH rats (Fig. 7). The relative proportions of type I, IIa, and IIc fibers were similar in all regions of normoxic rats (Fig. 5). However, in region 1 of CH rats, the proportion of type I fibers was significantly lower than in normoxic rats, and there was a trend for the proportion of IIa fibers to be higher (P = 0.061; Fig. 5). Similar trends were apparent in regions 2 and 3, but they did not reach statistical significance. The average FCSA was greater in region 1 than in regions 2 or 3 in both normoxic and CH rats, but there were no significant differences between the normoxic and CH rats in any region (Fig. 7).

Type I fibers were substantially larger in region 1 than in regions 2 and 3 in both normoxic and CH rats. The FCSA of type IIa and IIc fibers were similar in all regions of normoxic rats (Fig. 7), and the modest difference between normoxic and CH rats in the FCSA of types IIa or IIc did not reach significance in any region. However, when type IIa fibers were averaged over all regions, their FCSA was significantly greater in CH than in normoxic rats (2,300 ± 80 vs. 1,918 ± 108 μm², P < 0.01).

Fig. 4. Regional effects of chronic hypoxia on capillary supply and fiber size within the diaphragm. A: capillary-to-fiber ratio; B: capillary density; C: mean FCSA. Values are means ± SE. **P < 0.01 vs. normoxia (control). Diaph, diaphragm.
For the TA muscle, the average FCSA of type I, IIA, and IIB fibers were not significantly different after chronic hypoxia compared with the control values of 1,129 ± 69, 1,372 ± 96, and 2,295 ± 184 μm², respectively. Within the TA muscle, C/F was greater in CH rats than in normoxic rats in the outer cortex, where both the FCSA and proportion of type IIB fibers were greatest (Fig. 8), but not in the inner core (cf. Fig. 7). CD followed this trend with a 12% increase in the cortex and a 5% decrease in the core. The relative proportions of the different types of muscle fibers were similar in core and cortex except that there were no type I fibers in the cortex in either normoxic or CH rats, with a correspondingly greater proportion of type IIB fibers in both normoxic and CH rats. There were no differences between normoxic and CH rats in the proportion of any muscle fiber type in either the core or cortex (Fig. 5). The average FCSA was substantially greater in the cortex than the core in both normoxic and CH rats, there being no significant difference between normoxic and CH rats in either part (Fig. 8). However, given the larger size of type IIB fibers, the relative area of glycolytic tissue was much higher in the cortex.

The C/F in CH relative to normoxic rats was ~30% greater in region 1 of the SOL muscle and only 17% greater in the TA cortex (cf. Figs. 7 and 8); the difference between groups being significantly different in both muscle regions (P < 0.05). Thus angiogenesis occurred in regions of high FCSA, but the percent increase in C/F was greater in the muscle region that was dominated by type I fibers than that dominated by type IIB fibers.

The largest fibers introduce the longest diffusion distances in muscle, which, interpolating from a pub-
lished relationship between fiber area and oxygen tension (26), suggests a critical value of <15 mmHg (<2 kPa) for hypoxia-induced angiogenesis (see DISCUSSION). The combined effect of fiber composition and capillary supply results in a decrease in calculated intracellular O$_2$ tension of between 20% (SOL muscle) and 60% (EDL muscle) when muscles are working at maximal O$_2$ consumption (Table 3). In regions with the greatest degree of angiogenesis (SOL region 1 and EDL region 4), this corresponds to values of 11–12 mmHg (1.6–1.5 kPa) in CH rats.

**DISCUSSION**

**Methological considerations.** The level and duration of hypoxia and the muscles analyzed in the present study were comparable to those used in other studies. Some previous studies (40, 43) used Sprague-Dawley rats, whereas the present and other studies (1, 6) used Wistar rats, but interstrain differences in physiology are modest. Although the body weight at the time of the terminal experiment was greater than in some previous studies (e.g., Refs. 39 and 43), we minimized the influence of allometry and maturity on analysis of muscle capillarity that may otherwise confuse interpretation of the effects of hypoxia (23, 39) (see below). It therefore seems likely that the disparity between the present findings and the essentially negative results of previous studies can largely be explained by the sampling techniques and statistical methods that were used for analysis.

It is clear from our analyses of hindlimb muscles that variables such as C/F, mean fiber size, and the FCSA of individual muscle fiber types can vary considerably between muscle origins. This is evident from the data presented in Figs. 7 and 8, which show regional differences in capillary density and fiber size within the core and cortex of TA and SOL muscles. The capillary-to-fiber ratio (A), capillary density (B), and mean FCSA (C) are shown for regions 1–3. Values are means ± SE. *P < 0.05 and **P < 0.001 vs. normoxia (control).
between different regions within the same muscle. Previous studies (39, 40, 43) appear to have chosen a single area for analysis at random or on the basis of different fiber types that are readily discernible. The influence of regional variation would then be lost in the grouped data from the whole muscle, thus reducing the ability to discriminate between groups of animals. This is illustrated by our analyses of the EDL and TA muscles, which showed no difference between normoxic and CH rats for C/F when all regions within the muscles were grouped together, but showed clear trends for a greater C/F in CH rats when the different regions were considered separately. We therefore suggest that the unbiased sampling protocol used in the present study allowed us to detect effects of chronic hypoxia on muscle capillarity that have been previously missed.

**Gross comparisons between muscles.** We initially chose to compare the effects of chronic hypoxia on the diaphragm, EDL, SOL, and TA muscles because they show a range of muscle activities in conscious animals. Although the workload of the diaphragm per breath may not change in chronic hypoxia (47), the respiratory frequency of CH rats is 30–40% greater than that of normoxic rats (45) and, hence, its workload per unit time must be greater in CH than in normoxic rats. Because the SOL muscle is a postural muscle, it is likely to show the same level of activity in both CH and normoxic rats. Finally, although behavioral arousal increases locomotor activity on acute exposure to hypoxia (27), in the long term, activity of the EDL and TA muscles are likely to be similarly low in both groups when living in a confined space. On this basis, we hypothesized that if growth of new capillaries is directly or indirectly dependent on metabolic factors (2) rather than mechanical factors (22), chronic hypoxia would induce a greater angiogenesis in the diaphragm and SOL muscles than in the EDL or TA muscles.

Consistent with this hypothesis, the average C/F and CD over the muscle cross section were 13–17% greater in the diaphragm and SOL muscles of CH than in normoxic rats, whereas both variables were similar in the EDL and TA muscles of the two groups. Sillau and Banchero (40) also reported that the average FCSA of the SOL muscle in weight-matched normoxic and CH rats were similar, and the C/F of the SOL muscles was somewhat higher in the CH than in normoxic rats: the lack of statistical significance may be attributed to the high variance of the sampling protocol (see above). In contrast, the increase in CD observed in the diaphragm of CH rats (43) was entirely attributable to a ~25% decrease in FCSA because there was no detectable change in C/F, which suggests a major difference with the present study in addition to differences in sampling technique. Snyder et al. (43) exposed rats to 10% O2 for 5 wk, and they achieved a hematocrit of 72%, whereas our CH rats were exposed only to 12% O2 for 3 wk and achieved hematocrit values of ~56% (45). Thus it may be that the additional stress of more severe hypoxia led to atrophy of the muscle fibers, as has been observed in human subjects during a prolonged period at high altitude where smaller fibers associated with loss of muscle protein resulted in an unchanged capillarity (21). Whether or not this is the case, our data showed that moderate systemic hypoxia does not cause obvious muscle atrophy, supported by our finding that the dry weights of the muscles were similar or greater in CH than in normoxic rats. However, it does cause angiogenesis, which is more pronounced in active muscles.

Nevertheless, initial hyperventilation may have acted as a mechanical trigger for angiogenesis, and factors in addition to muscle activity may play a role in determining the extent of hypoxia-induced angiogenesis. Because the EDL muscle is mainly composed of type IIb with few type IIa and type I fibers, whereas the SOL muscle has a high proportions of type IIa and I fibers, there was the possibility that angiogenesis was more likely to occur in muscles, or regions of muscles, with a high oxidative capacity. On the other hand, the observation that the difference between CH and normoxic rats tended to be greater in some regions raised the possibility that angiogenesis occurs preferentially where fibers are large. More detailed analyses were used to test these possibilities.

**Regional comparisons within and between muscles.** Fiber type compositions of the SOL and TA muscles were very different and showed regional differences in FCSA, whereas the TA, like EDL, muscles might be expected to be relatively inactive compared with the SOL muscle in rats living in a confined space. Although the average FCSA across all muscle fiber types varied between regions, they were not different between normoxic and CH rats for any region within any of these muscles, or regions of muscles, with a high oxidative capacity. That C/F was significantly greater in CH than in normoxic rats in region 1 of the SOL muscle and in the cortex of the TA muscle, where the fibers were largest (average FCSA 2,750 and 2,800 μm², respectively, cf. region 3 of the SOL muscles and in core of the TA muscle, where FCSA were only 2,150 and 1,375 μm², respectively), is consistent with the possibility that the degree of hypoxia-induced angiogenesis is determined by fiber size. If this hypothesis is correct, then the fact that C/F was also increased in region 2 of the SOL muscle, where the FCSA was 2,400 μm², suggests either that the average FCSA of these fibers was greater than some critical value or that some angio-

<table>
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<th>Table 3. Calculated intracellular O₂ tension in fibers from normoxic and CH rats at maximal O₂ consumption</th>
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<tr>
<td>SOL muscle</td>
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<td>Region 1</td>
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<td>Region 4</td>
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<td>TA muscle</td>
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Values are means ± SD. Intracellular O₂ tension was measured in kilopascals (kPa). Values in parentheses are minimum values.
genic factor diffused from region 1 (see below). Furthermore, because the type IIb fibers of the diaphragm were large and evenly distributed within the muscle, any development of intracellular hypoxia would be expected to lead to release of a homogeneous metabolic stimulus for angiogenesis. Thus it is consistent with our hypothesis that only modest regional differences were seen in muscle capillary supply and fiber size (Fig. 4).

Sillau and Banchero (40) found no difference in composition of the SOL muscle and only a 3% increase in the proportion of type IIb fibers in the TA muscle of CH rats. In the present study, little or no change was seen in the TA muscle either at a gross or regional level, and only region 4 of the EDL muscle showed a significant reciprocal increase in type IIa and a decrease in type IIb fibers. On the other hand, Itoh et al. (24) and Ishihara et al. (23) reported that chronic hypoxia caused a relative increase in the proportion of type IIa fibers in the SOL muscle by inhibiting the normal conversion of type IIa to I fibers during maturation. If their data are compared at the same body weight rather than the same age, then the difference between the proportions of type I fibers in the two groups of rats are very small. This is consistent with the present results, where the proportion of type I fibers tended to be smaller in the SOL muscle of CH rats, reaching statistical significance in region 1. Interestingly, angiogenesis occurred in both region 1 of the SOL muscle and region 4 of the EDL muscle in CH rats, where the proportion of type IIa [the fiber type with the highest oxidative capacity in rodent muscle (14)] fibers was increased.

Thus any effect chronic hypoxia has on muscle fiber type, whether the muscle as a whole has high oxidative or high glycolytic activity, is clearly very small and unlikely to have contributed to the hypoxia-induced angiogenesis. Indeed, experiments involving exercise training or chronic electrical stimulation of muscle suggest that increases in C/F of the order seen in the present study should be associated with substantial increases in the proportion of fibers with high oxidative activity if they were causally related (22). Moreover, any differences between the proportions of muscle fiber types were also unlikely to explain why angiogenesis occurred in some regions of each muscle but not others. However, region 1 of the SOL muscle did show a much larger increase in C/F than the cortex of the TA muscle (30 vs. 17%) in CH versus normoxic rats. Thus, although muscle fiber type is not a major factor in determining whether or not angiogenesis occurs within skeletal muscle in chronic hypoxia, it may facilitate some factor that is related to muscle fiber size. This hypothesis is supported by the finding that within the SOL, TA, and EDL muscles, the FCSA of individual fiber types showed regional variation such that the dominant type I fibers of the SOL muscle were substantially larger in region 1, whereas the dominant type IIb fibers of the TA and EDL muscles were substantially larger in the cortex and in region 4, respectively: the very regions in which increases in C/F occurred.

Intercapillary distance has been used for many years as an index of the diffusive limits to peripheral oxygen transport, but it seems to be a relatively insensitive descriptor of the changes we observed (Fig. 9). We therefore adopted an alternate approach by calculating muscle oxygen tension. Leon-Velarde et al. (26) used Krogh’s equation as modified by Kety to graphically represent the tissue PO2 as a function of the radial distance from a capillary. With the use of this approach with fiber diameter as an index of intercapillary distance and values for O2 transport in muscle as estimated by Weibel (51), we can estimate tissue PO2 for the various muscle regions studied at venous PO2 values of 40 and 30 mmHg (5.3 and 4 kPa), values actually measured in normoxic rats breathing air and in normoxic and CH rats breathing 12% O2, respectively (28). At a venous PO2 of 40 mmHg, regions 1 and 2 of the
SOL muscle, region 4 of the EDL muscle, and the cortex of the TA muscle would all have tissue PO2 values of 15–20 mmHg (2–2.7 kPa), which would have dropped to <12 mmHg (1.6 kPa) when venous PO2 fell to 30 mmHg, assuming no increase in C/F. In contrast, region 3 of the SOL muscle, regions 1, 2, and 3 of the EDL muscle, and the core of the TA muscle would all have tissue PO2 values of >22 mmHg (2.9 kPa) when breathing air, whereas tissue PO2 would have remained at >14 mmHg (1.9 kPa) under hypoxia, again assuming no increase in C/F. This suggests that the critical tissue PO2 value for triggering capillary angiogenesis may lie between 10 and 15 mmHg (1.3–2 kPa).

To explore the potential influence that the observed changes in muscle composition would have on muscle O2 tension, we also used a model of O2 diffusion to calculate the mean intracellular PO2 of different fiber types based on capillary supply, fiber size, and mitochondrial content (20). These data suggest that at maximal O2 consumption, individual fibers would have developed values of ~12 mmHg (1.6 kPa), which may have been an adequate stimulus for the capillary growth observed in SOL and EDL muscles (Table 3) but could not explain the angiogenesis in the TA cortex, where the relatively low O2 consumption would maintain a much higher PO2 of 31 mmHg (4.2 kPa). This suggests that chronic hypoxia rather than muscle activity was the dominant stimulus for angiogenesis and that, if a high level of muscle activity was also involved, we might have seen a different pattern of angiogenesis to that actually observed.

Stimulus for angiogenesis. Angiogenesis in skeletal muscle is thought to be triggered by local mechanical or metabolic factors, which may in turn trigger the release of peptide growth factors (2, 22). Pertinent mechanical factors may include vessel strain imposed by large fiber diameter as increased tension in the blood vessel walls or increased shear stress in their lumen (22). The acute response to systemic hypoxia in the rat includes a fall in arterial pressure and an increase in muscle vascular conductance, indicating vasodilatation, such that gross blood flow remains constant (28). This tonic response is maintained at least during the first 3–7 days of chronic systemic hypoxia, when the trigger for angiogenesis would be expected to be present, although values subsequently returned toward those of normoxic rats (50). It is therefore unlikely that either wall tension or luminal shear stress are elevated during this early period across the whole muscle, although there may be regional variations within muscle. Similarly, it is possible that shear stress was increased by the rise in hematocrit caused by chronic hypoxia, although polycythemia per se failed to elicit angiogenesis in rat muscle (10), and, over the first 7–8 days, the increase in hematocrit was modest (41). On this basis, it is likely that in chronic hypoxia angiogenesis is triggered primarily by metabolic factors, consistent with the theoretical analysis (see above) of its effect on muscle PO2 at rest and at maximum activity.

Of the growth factors that have been implicated in angiogenesis, vascular endothelial growth factor (VEGF) is recognized to be upregulated by hypoxia both in vivo and in vitro (33, 38). Expression of VEGF mRNA and amount of VEGF protein has been shown to increase in skeletal muscle of rats within a few hours of the onset of hypoxia (32) and when exercise is performed during hypoxia (8), although this was not confirmed by Gustafsson et al. (18). Adenosine has also been implicated in the endothelial cell proliferation that occurs in hypoxia (29) and has been shown to increase the expression of VEGF in endothelial cells and vascular smooth muscle (17, 36). Adenosine is predominantly released from the vascular endothelium during acute systemic hypoxia (30), which is likely to be most pronounced in regions where the FCSA of the muscle fibers and the diffusional distances for O2 are largest. This may be accentuated where the muscle fibers are active and particularly where the fibers are oxidative rather than glycolytic (7). Release of adenosine would then not only cause dilatation of the arterioles to offset the tissue hypoxia by increasing blood flow but also trigger the synthesis of VEGF. By inducing capillary angiogenesis (present study) and remodeling of the arteriolar tree (41), the improved O2 distribution would then relieve tissue hypoxia. Subsequently, regions of muscle where angiogenesis does not occur may then be subject to a suppressive influence, such as the potent inhibitor of endothelial cell proliferation transforming growth factor-β, which is also upregulated during hypoxia (49). Our observations of capillary growth in the continually active diaphragm muscle, in the regions with large oxidative fibers of the tonically active SOL muscle, and also in the large fiber regions of the relatively inactive TA and EDL muscles are thus consistent with the metabolic control of angiogenesis in chronic systemic hypoxia.

In conclusion, this study has shown for the first time that chronic systemic hypoxia lasting 3 wk resulted in a greater capillarity in the more active muscles of the rat irrespective of fiber area viz. diaphragm and SOL muscle. As the higher CD was not associated with a lower average FCSA, we conclude that the greater C/F in muscles of CH rats therefore reflected true angiogenesis. Because the EDL and TA muscles are predominantly composed of fast glycolytic fibers, whereas the SOL muscle is mainly composed of slow oxidative fibers, it is clear that chronic hypoxia-induced angiogenesis in skeletal muscles that are metabolically very different. Thus whether or not chronic hypoxia induced capillary angiogenesis within a given muscle was apparently dependent on the size of muscle fibers, although where FCSAs were similar the amount of angiogenesis was greater in muscles with a higher oxidative capacity.

**Perspectives.** Changes in muscle fiber composition caused by oxidative stress associated with increased muscle activity are accompanied by changes in muscle capillarity (22), suggesting that similar changes may occur in chronic hypoxia. Previously, direct measurements of PO2 showed no evidence for tissue hypoxia as
the angiogenic stimulus, although increases in blood flow may act as an important stimulus for growth of capillaries. For example, myocardial capillary growth in animals exposed to high-altitude hypoxia occurs only in the right but not the left ventricle, and blood flow is increased in the right ventricle, which has to work harder to overcome the increased pulmonary resistance, but not in the left (46). Similarly, expansion of the volume of microvessels by elongation of existing cells in the brains of rats exposed to hypobaric hypoxia (25) may be explained on the basis of increased blood flow. Our data suggest that chronic hypoxia results in an integrated stimulus for capillary growth. The metabolic type and activity level of muscle influences the degree of hypoxia-induced angiogenesis, with the size of fiber either contributing directly via production of a metabolic stimulus or indirectly via a mechanical stimulus. These conclusions are consistent with those regarding the influence of fiber size on muscle capillarity during ontogenetic growth in a range of vertebrates (see Ref. 15), including humans (3), and during the adaptive remodeling during chronic cold exposure (12) or differential muscle activity (16). Chronic systemic hypoxia may therefore be a useful model to examine the mechanism of changing functional O2 supply under conditions of unchanging oxygen demand, in contrast to the situation with altered muscle activity, where changes in peripheral O2 transport correspond with increased O2 demand.

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REFERENCES


32. Partovian C, Adnot S, Eddahibi S, Teiguer E, Levanne M, Dreyfus P, Raffestin B, and Frelin C. Heart and lung VEGF mRNA expression in rats with monocrotaline- or hypoxia-in


