Region-specific adaptations in determinants of rat skeletal muscle oxygenation to chronic hypoxia

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1Institute for Biomedical Research into Human Movement and Health, Manchester Metropolitan University, Manchester; 2Research Institute MOVE, Faculty of Human Movement Sciences, Vrije Universiteit Amsterdam, Amsterdam; 3Department of Pediatrics and 4Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen; and 5Department of Physiology, Institute for Cardiovascular Research, VU University Medical Centre, Amsterdam, The Netherlands

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Wüst RC, Jaspers RT, van Heijst AF, Hopman MT, Hoofd LJ, van der Laarse WJ, Degens H. Region-specific adaptations in determinants of rat skeletal muscle oxygenation to chronic hypoxia. Am J Physiol Heart Circ Physiol 297: H364–H374, 2009. First published May 8, 2009; doi:10.1152/ajpheart.00272.2009.—Chronic exposure to hypoxia is associated with muscle atrophy (i.e., a reduction in muscle fiber cross-sectional area), reduced oxidative capacity, and capillary growth. It is controversial whether these changes are muscle and fiber type specific. We hypothesized that different regions of the same muscle would also respond differently to chronic hypoxia. To investigate this, we compared the deep (oxidative) and superficial (glycolytic) region of the plantaris muscle of eight male rats exposed to 4 wk of hypobaric hypoxia (410 mmHg, P2: 11.5 kPa) with those of nine normoxic rats. Hematocrit was higher in chronic hypoxic than control rats (59% vs. 50%, P < 0.001). Using histochemistry, we observed 10% fiber atrophy (P < 0.05) in both regions of the muscle but no shift in the fiber type composition and myoglobin concentration of the fibers. In hypoxic rats, succinate dehydrogenase (SDH) activity was elevated in fibers of each type in different regions of the muscle but no shift in the fiber type composition. We hypothesized that different regions of the same muscle would also respond differently to chronic hypoxia. To investigate this, we compared the deep (oxidative) and superficial (glycolytic) region of the plantaris muscle of eight male rats exposed to 4 wk of hypobaric hypoxia (410 mmHg, P2: 11.5 kPa) with those of nine normoxic rats. Hematocrit was higher in chronic hypoxic than control rats (59% vs. 50%, P < 0.001). Using histochemistry, we observed 10% fiber atrophy (P < 0.05) in both regions of the muscle but no shift in the fiber type composition and myoglobin concentration of the fibers. In hypoxic rats, succinate dehydrogenase (SDH) activity was elevated in fibers of each type in the superficial region (25%, P < 0.05) but not in the deep region, whereas in the deep region but not the superficial region the number of capillaries supplying a fiber was elevated (14%, P < 0.05). Model calculations showed that the region-specific alterations in fiber size, SDH activity, and capillary supply to a fiber prevented the occurrence of anoxic areas in the deep region but not in the superficial region. Inclusion of reported acclimatization-induced increases in mean capillary oxygen pressure attenuated the development of anoxic tissue areas in the superficial region of the muscle. We conclude that the determinants of tissue oxygenation show region-specific adaptations, resulting in a marked differential effect on tissue P2.

An increase in capillary density (CD) in rat muscle after 6 wk of exposure to 12% oxygen (21, 22) may serve to facilitate oxygen supply to muscle cells by reducing the diffusion distances of and exchange area for oxygen. This response is realized by angiogenesis (21, 22) and/or a decrease in fiber cross-sectional area (FCSA) (3, 25). A further improvement of the oxygen supply to the mitochondria might be realized by an increase in myoglobin (Mb) concentration enhancing the facilitation of intracellular diffusion of oxygen (60). These results, however, are not consistent as unaltered FCSA (21, 67), capillarization (1, 65), and/or Mb concentration in response to chronic hypoxia have also been reported (41, 66).

Besides adaptations in capillarization, chronic hypoxia might also affect energy metabolism. In both human and rat muscles, a decreased oxidative capacity has been reported and is accompanied by an increased glycolytic capacity (1, 12, 30, 38, 39, 48). Others, however, have reported that the oxidative capacity is unchanged (47) or even increased after exposure to hypoxia (26, 33).

The observation that adaptations to hypoxia are muscle specific and even specific to the region within a muscle could explain these discrepancies (22, 24, 55, 66, 67). Indeed, it has been reported that slow and fast muscles respond differently to chronic exposure of hypoxia (22, 24). Even within one muscle with a nonuniform fiber type distribution, the capillary adaptation to hypoxia seems to depend on the region of the muscle, where the hypoxia-induced angiogenesis was greater in regions within a muscle with a lower oxidative capacity (22).

To investigate whether adaptations to chronic hypoxia are fiber type and/or region specific, rats were exposed for 4 wk to chronic hypobaric hypoxia (410 mmHg). We hypothesized that 1) overall, there is atrophy and a decrease in oxidative capacity; 2) in the superficial region of the muscle, angiogenesis is more pronounced than in the deep region; and 3) these adaptations prevent the occurrence of anoxic areas in both regions of the muscle during hypoxia.

We used an integrative approach to assess the skeletal muscle fiber adaptations to chronic hypoxia by determining changes in fiber type, size, capillary supply, oxidative capacity, and Mb concentration in muscle fibers of distinct oxidative and glycolytic regions in serial histological sections of the plantaris muscle of the rat. The functional consequences of these adaptations were calculated using a Krogh model (36, 46) and a Hill model (19, 32).
METHODS

Animals

Seventeen male Wistar rats, aged 8 wk at the start of the study, were used. Experimental rats (n = 8) were housed in a hypobaric hypoxic chamber at a barometric pressure of 410 mmHg and 20.9% O2 [equivalent to a PO2 of 11.5 kPa (86 mmHg) and that experienced at ~5,000 m of altitude]. Normobaric rats (n = 9) lived in the same room but at ambient pressure (~760 mmHg). All rats were kept at room temperature and a 12:12-h light-dark cycle. Food and water were given ad libitum, and the cage was opened for 30 min maximally once a week for cleaning. After 4 wk of exposure to hypobaric hypoxia or normobaric conditions, rats were anesthesia by an intraperitoneal injection of pentobarbital sodium (70 mg/kg), and the right plantar muscle was dissected from the surrounding tissue, blotted dry, and weighed. The muscle was then pinned on cork and slightly stretched above its slack length to minimize any bias in the determination of FCSA and capillary parameters, related to differences in muscle length, quickly frozen in liquid nitrogen, and stored at ~80°C until further analysis. All protocols and procedures were approved by the University of Nijmegen Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Hematocrit and Hemodynamic Measurements

Hematocrit levels were determined in blood samples taken from the right orbita. Right ventricular systolic pressure was measured (MIDAC software, Radboud University, Nijmegen, The Netherlands) with an ultaminiature catheter (model SPR-320, Millar Instruments, Houston, TX) attached to a pressure transducer introduced into the right external jugular vein and placed in the right ventricle by pressure tracing. The right ventricle was dissected from the heart, weighed, and the weight expressed as a percentage of the total body weight.

Myosin Heavy Chain Composition

To determine the myosin heavy chain (MHC) composition (type I, IIA, IIX, and IIB) of the plantaris muscle, we used a modification of the SDS-PAGE method used previously (13). In short, a 10-µm section was dissolved in 300 µl Laemmlli buffer, and 11 µl were loaded onto 6% acryl amide gels. Gels were run at 250 V for 25 h and subsequently stained with a Silverstain Plus Kit (Bio-Rad, Hemel Hempstead, UK).

Staining of Histological Sections

Serial 10-µm cross sections from the middle of the plantaris muscle were cut on a cryostat at −20°C and mounted on polylysine-coated slides. Sections were stored at −80°C until further analysis unless otherwise stated. All sections were mounted in glycerin-gelatin after being stained.

Myosin ATPase

Sections were stained for myosin ATPase to classify fibers as type I, Ila, and IIX/b as previously described (8).

Succinate Dehydrogenase Activity

Succinate dehydrogenase (SDH) activity in individual muscle cells in histological sections was determined immediately after sectioning as previously described (57). Sections were incubated at 37°C in the dark for 20 min in medium consisting of 37 mM sodium phosphate buffer (pH 7.6), 74 mM sodium succinate, and 0.4 mM tetranitroblue tetrazolium. After incubation, the reaction was stopped in 0.01 M HCl. The staining intensity of SDH was determined by measuring the absorbance of the final reaction product using an interference filter at 660 nm. Absorbances were converted to the rate of staining and expressed as absorbance units (ΔA₆₆₀) per micrometer section thickness per second of incubation time (ΔA₆₆₀·µm⁻¹·s⁻¹). The staining rate is proportional to the maximum rate of oxygen uptake by the muscle cell when oxygen is not rate limiting (76).

Mb Concentration

The Mb concentration in individual muscle cells was determined as previously described (74). In short, frozen sections were defrosted in a vacuum to avoid the condensation of water on the sections and hence the redistribution of Mb. Sections were then vapor fixed for 1 h at 70°C in paraformaldehyde and subsequently, sections were fixed for 10 min at room temperature in 2.5% glutaraldehyde in 0.07 M sodium phosphate buffer (pH 7.4) and quickly rinsed with distilled water. To detect Mb, sections were incubated for 1 h at room temperature in medium containing 50 ml of 50 mM Tris and 80 mM KCl buffer (pH 8.0), 25 mg ortho-toluidine dissolved in 2 ml of 96% ethanol (at ~50°C), and 1.43 ml of 70% tertiary-butyl hydroperoxide. Sections were then washed with distilled water and mounted. Calibrated images were studied on a DMRB microscope (Leica, Wetzlar, Germany) at 436 nm.

Capillarization

Capillaries were depicted by staining sections for alkaline phosphatase as previously described (18, 21, 22). The capillarization was analyzed using the method of capillary domains (18, 37). This method does not only provide the overall indexes of capillary density (CD) and capillary-to-fiber ratio but also provides measures of the capillary supply to individual fibers, even when they lack direct capillary contacts, and allows the calculation of capillary supply to different fiber types. Also, an index for the heterogeneity in capillary spacing can be calculated, which is an important factor for tissue oxygenation (14). Since the deep and superficial regions of the muscle differ in their fiber type composition and capillary supply, we analyzed these regions separately. In short, photomicrographs of cross sections of the deep and superficial regions of the plantaris muscle were taken. The fiber type distribution was assessed, and the outlines of the muscle fibers as well as the location of each capillary were digitized (model MMII 1201, Summagraphics) and fed into the computer program. The overall CD was defined as the number of capillaries per millimeter squared of tissue. Capillary domains, defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent ones, were constructed, and their surface area was calculated. The SD of the log-transformed domain area (logSD) gives an indication of the heterogeneity of the capillary spacing. A decrease in this parameter indicates a more homogeneous distribution of capillaries. From the overlap of the capillary domains with muscle fibers, the local capillary-to-fiber ratio (LCFR) and the capillary fiber density (CFD) were obtained. The LCFR for a fiber was defined as the sum of the fractions of each domain area overlapping the fiber. This variable has a continuous distribution and takes into account remote capillaries, thus allowing the determination of the capillary supply to a fiber even when it lacks direct capillary contacts. Such situations were observed occasionally in the glycolytic region of the plantaris muscle. CFD, which is LCFR divided by FCSA, provides the CD of that fiber and is expressed as the number of capillaries per millimeter squared.

Modeling of Skeletal Muscle Tissue Oxygenation

We applied the Hill model and an extended Krogh model to obtain some insight as to how the observed adaptations affected tissue oxygenation during hypoxia. Both models assume that oxygen diffuses over the cell membrane and through the cytoplasm into the mitochondria and take into account Mb-facilitated oxygen diffusion. The Hill model assumes homogeneous Po2 in the intracellular space around the fiber, whereas the Krogh model assumes that oxygen
diffuses from point sources of oxygen (capillaries). We assumed that at the maximum oxygen consumption of the cell (VO_{2max}), the flow through and PO_{2} in all capillaries are the same.

*Hill model.* The critical extracellular PO_{2} required to prevent the development of an anoxic core in a cylindrical cell (PO_{2, crit; in mmHg}) at V_{O_{2} max} (in mM/s) was calculated with the following Hill model (32, 54):

\[ \text{PO}_{2, \text{crit}} = \left( \frac{\text{V}_{O_{2} \text{max}} - \text{FCSA}}{4\text{P} \cdot \Delta \text{Mb} \cdot [\text{MbO}_{2}]/[\text{Mb}]} \right)^{1/4} \text{P} \cdot \text{O}_{2} \cdot \text{DO}_{2} \]

where V_{O_{2} max} was calculated using SDH activity (76), FCSA was measured in millimeters squared, \(\Delta\text{Mb}\) is the radial diffusion coefficient of Mb [0.27 \times 10^{-4} mm²/s (4)], [MbO₂]/[Mb] is the concentration of oxygenated Mb at the sarcolemma (in mM) and was calculated from the total Mb concentration in individual fibers using methods described in Des Tombe et al. (19), \(\alpha_M\) is the solubility of oxygen in skeletal muscle, and DO₂ is the diffusion coefficient for oxygen in skeletal muscle. The product of the latter two (\(\alpha_M \cdot \text{DO}_{2}\)) is known as Krogh's diffusion coefficient (2 nM·mm⁻²·mmHg⁻¹·s⁻¹) (75).

*Krogh model.* The critical PO₂ just outside the capillary required to prevent the development of anoxic tissue areas when the muscle is working at V_{O_{2} max} (PO_{2; cap; in mmHg}) was calculated using a universal Krogh's tissue model (36). We used photomicrographs of sections with a representative capillarization for each region and condition. For the model calculations, the capillary radius was set at 2.4 μm. The weighted average VO_{2max} and Mb concentration were fed into the model. Where the Hill model calculates a PO₂ that should be uniformly present around a muscle fiber, Krogh's tissue model calculates a minimal PO₂ at the capillary that ensures the absence of anoxic areas.

We also used the Krogh's tissue model to estimate the distribution of oxygen pressure within the muscle tissue during normoxia (mean capillary PO₂ of 55 mmHg) and acute hypoxia (mean capillary PO₂ of 25 mmHg, with both conditions using tissue characteristics of the normoxic situation). We then fed into the model the observed skeletal muscle adaptations to chronic hypoxia and assessed how these affected the PO₂ distribution in the tissue during hypoxia (mean capillary PO₂ of 25 mmHg). Finally, we fed into the model not only the observed skeletal muscle adaptations to chronic hypoxia but also the reported increase in mean capillary PO₂ [35 mmHg (9)] to see how this would affect the tissue PO₂ distribution.

**Statistical Analysis**

Independent t-tests were used to compare means between the two groups. Multilevel analysis (MLwiN, version 2.02, Centre for Multi-level Modeling, Bristol, UK) was used to test for significant differences in capillarity, oxidative capacity, Mb concentration, and fiber size between region, fiber type, and hypoxia/normoxia. Multilevel analysis can be considered as an extension to the commonly used linear regression analysis, which has the disadvantage that only a continuous outcome variable can be analyzed (73). Moreover, it incorporates variance at the level of the cell and the individual. Differences were considered significant at P < 0.05. Unless stated otherwise, values are means ± SD.

**RESULTS**

Body weights of hypoxic (363 ± 40 g) and control (383 ± 28 g) rats were not significantly different. In addition, weights of control and hypoxic plantaris muscles (397 ± 76 vs. 370 ± 31 mg, respectively) did not differ significantly. Ratios of plantaris muscle mass to body mass were also similar in control and hypoxic rats: 1.04 ± 0.17 and 1.02 ± 0.05 mg/g, respectively. That the rats were really hypoxic was reflected by the higher hematocrit than control rats (49.9 ± 0.8 vs. 58.9 ± 0.9%, P < 0.001) as well as elevated right ventricular systolic pressure (from 15 ± 32 to 74 ± 11 mmHg in controls vs. hypoxic rats, P < 0.001), and right ventricular hypertrophy (from 0.75 ± 0.15 to 1.02 ± 0.12 g/body wt, P < 0.001), similar to that reported previously in hypobaric hypoxia (80).

In total, 990 control and 1,130 hypoxic fibers were analyzed. For each fiber, all parameters were assessed in serial muscle sections; a typical example is shown in Fig. 1.

**Fiber Type Composition and FCSA**

The overall MHC composition of the plantaris muscle did not change significantly after exposure to hypoxia (Fig. 2). The deep region had a higher proportion of type I fibers and a lower proportion of type IIX/b fibers than the superficial region (P < 0.001), whereas there were no significant regional differences in the proportion of type IIa fibers. In line with the MHC data, hypoxia did not result in an altered fiber type composition (Fig. 3A).

In both regions, the size of the fibers was in the order of I < IIa < IIX/b (P < 0.05). Type I fibers in the deep region were larger and type IIX/b fibers were smaller than those in the superficial region (P < 0.05), whereas type IIa fibers had a similar size in both regions. Hypoxia resulted in an overall 10% reduction in FCSA (P < 0.05; Fig. 3B). The absence of an interaction between fiber type and/or region with hypoxia indicates that this effect was similar for each fiber type and independent of region.

**SDH Activity**

SDH activity in type IIX/b fibers was lower than that in the other fibers (P < 0.001; Fig. 4A). Interestingly, in the deep region only, the SDH activity of type IIa fibers was higher than that of type I fibers (P < 0.001). Overall, the SDH activity was 26% lower in the superficial region than in the deep region (P < 0.001). This was explicable by the larger number of type IIX/b fibers in the superficial region than in the deep region. In addition, the SDH activity in type IIX/b fibers in the superficial region was lower than that in the deep region (P < 0.001), whereas there were no significant differences in activity in type I and IIa fibers between the two regions. Hypoxia induced an overall 25% increase in the SDH activity in fibers in the superficial region (P < 0.05), whereas no change in the SDH activity occurred in the deep region.

Spatially integrated SDH activity is proportional to the VO_{2max} per millimeter of fiber length (in nmol·mm⁻¹·s⁻¹) of the cell (76). In control rats, type I fibers had a lower integrated SDH activity compared with type II fibers (IIa and IIX/b fibers, P < 0.001), whereas there were no differences between IIa and IIX/b fibers (Fig. 4B). Integrated SDH activity was higher in fibers from the deep region compared with those from the superficial region (P < 0.001). This was due to a higher integrated SDH activity of type II fibers (P < 0.001) but not of type I fibers in the deep region than in the superficial region of the muscle. After exposure to hypoxia, this difference between the two regions disappeared (P = 1.00).

**Mb Concentration**

Overall, fibers in the superficial region of the plantaris muscle had a 30% lower Mb concentration than those in the deep region (P < 0.001; Fig. 5). The Mb concentration of type IIa fibers in both the deep and superficial regions was higher than that of type I fibers (P < 0.05), whereas type IIX/b fibers had the lowest Mb...
concentration ($P < 0.001$). Hypoxia did not significantly affect the Mb concentration in fibers of any type or region.

**Capillarization**

The LCFR for each fiber type and the overall capillary-to-fiber ratio (shown as “total”) are shown in Fig. 6A. In both regions, the LCFR of type IIx/b fibers was higher than that of the other fibers ($P < 0.001$). In the deep region, type I and IIa fibers had a similar LCFR, whereas in the superficial region, the LCFR of type IIa fibers was higher compared with type I fibers ($P < 0.001$). The LCFR for each fiber type was higher in the deep region compared with the superficial region ($P < 0.001$). There was an interaction between region and hypoxia ($P < 0.001$), which was apparent as a 14% increase in the LCFR after hypoxia of fibers in the deep region ($P < 0.05$) but not in the superficial region.

The CFD of fibers of each type was lower in the superficial region than in the deep region ($P < 0.001$; Fig. 6B). The CFD of type IIx/b fibers was lower than those of type I and IIa fibers ($P < 0.001$), which had a similar CFD. Hypoxia resulted in a 24% higher CFD in the deep region ($P < 0.01$) but not the superficial region of the muscle.

The heterogeneity of capillary spacing ($\log_{10} SD$) was larger in the superficial region compared with the deep region (0.097 ± 0.013 vs. 0.112 ± 0.011, means ± SE, $P < 0.001$) but was not significantly changed by hypoxia in either region (0.091 ± 0.010 and 0.118 ± 0.022 for the deep and superficial regions, respectively).

**Relationships Among Capillarization, SDH Activity, and FCSA**

To investigate how the adaptations in response to hypoxia affected oxygen supply and demand, we calculated the relationships among LCFR, SDH activity, and FCSA for all fiber types. Relations for type IIa and IIx/b fibers, as they are the predominant fibers in the plantaris muscle, are shown in Fig. 7.

As expected, LCFR was linearly related to the maximal oxygen uptake of the cell (as reflected by the spatially inte-
grated SDH activity) in type I fibers ($R^2$ ranged from 0.165 to 0.640 for each region and condition, $P < 0.001$), type IIA fibers ($R^2$ ranged from 0.110 to 0.336, $P < 0.001$; Fig. 7A), and type IIX/b fibers ($R^2$ ranged from 0.070 to 0.284, $P < 0.001$; Fig. 7B) except for type IIX/b fibers in the normoxic superficial region ($R^2 = 0.012, P = 0.06$). After hypoxia, the correlation coefficient and slope of this relation increased for type IIX/b fibers in both regions ($P < 0.001$).

There was no significant relationship between LCFR and SDH activity for type I fibers ($R^2$ ranged from 0.000 to 0.144) and IIA fibers ($R^2$ ranged from 0.008 to 0.052; Fig. 7C), and in type IIX/b fibers in the superficial region, a slight negative relationship was observed ($R^2 = 0.018, P < 0.01$; Fig. 7D, dotted line), which disappeared ($P < 0.03$) after exposure to hypoxia ($R^2 = 0.000$).

Whereas no correlation was found between LCFR and SDH activity, LCFR did not significantly correlate positively with FCSA in type I fibers ($R^2$ ranged from 0.226 to 0.487), IIA fibers ($R^2$ ranged from 0.115 to 0.491; Fig. 7E) and IIX/b fibers ($R^2$ ranged from 0.127 to 0.341; Fig. 7F). For all fiber types, the slope between FCSA and LCFR significantly increased in the deep region ($P < 0.05$) but not in the superficial region after hypoxia. Interestingly, whereas the region-specific difference in the slope was not significant in normoxia, hypoxia amplified the difference in the slope between the deep and superficial regions for all fiber types, which was entirely due to an increased slope in the deep region only. This indicated that at a given FCSA, type I, IIA, and IIX/b fibers in the superficial region had a similar LCFR, whereas those in the deep region had a higher LCFR after hypoxia ($P < 0.001$). This was reflected by the increased CFD for each fiber type in the deep region but not in the superficial region during hypoxia (Fig. 6B). Overall, the CFD of type I fibers was higher than that for type IIA fibers. Type IIX/b fibers had the lowest CFD ($P < 0.001$).
Model calculations were used to assess the functional consequences of the observed adaptations. During normoxia, the $P_{O_2 \text{crit}}$ for each individual muscle fiber (Hill model) was higher in the deep region compared with the superficial region ($P < 0.001$; Fig. 8A). In response to chronic hypoxia, the regional difference in $P_{O_2 \text{crit}}$ disappeared, due to a decrease in the deep region and an increase in the superficial region (interaction between hypoxia and region, $P < 0.001$). The decrease in $P_{O_2 \text{crit}}$ in the deep region was caused by muscle fiber atrophy, whereas the increase in the superficial region resulted from an increase in SDH activity.

Subsequently, we calculated the $P_O_2$ that is required in the capillaries to prevent the occurrence of any anoxic area at $V_{O_2\max}$ (as was calculated for $P_{O_2 \text{crit}}$), which is referred to as $P_{O_2 \text{cap}}$. To this end, we chose photographs with representative capillary distributions for each region and condition. $P_{O_2 \text{cap}}$ in the superficial region was higher compared with the deep region. $P_{O_2 \text{cap}}$ in the deep region did not differ between normoxia and hypoxia, but hypoxia resulted in a 25% increase in $P_{O_2 \text{cap}}$ in the superficial region of the muscle (Fig. 8B).

The $P_O_2$ distribution, shown as a cumulative curve (in %), in the muscle tissue at $V_{O_2\max}$ was calculated using the Krogh model in normoxia [Fig. 9A; mean capillary $P_O_2$ set to 55 mmHg (9)], acute hypoxia [Fig. 9B; mean capillary $P_O_2$ set to 25 mmHg (9)], and after chronic exposure to hypoxia (mean capillary $P_O_2$ set to 25 mmHg), taking into account skeletal muscle adaptations (Fig. 9B). Figure 9C shows the distribution of tissue $P_O_2$ when not only the adaptations in the skeletal muscle but also the reported changes in mean capillary $P_O_2$ (presumably due to increases in hematocrit and blood flow) were fed into the model (mean capillary $P_O_2 = 35$ mmHg). While during normoxia no anoxic regions were present in either the deep or superficial regions of the muscle (Fig. 9A), acute hypoxia (25 mmHg) resulted in a marked leftward shift in the $P_O_2$ distribution in the tissue; during acute hypoxia, ~75% of the skeletal muscle tissue in the superficial region was anoxic and 40% of the skeletal muscle tissue in the deep region was anoxic. The adaptations of the skeletal muscle tissue to chronic hypoxia resulted in a marked region-specific adaptation: whereas in the deep region the adaptations in the skeletal muscle (higher capillarization and muscle atrophy) resulted in a rightward shift compared with the acute hypoxic situation, indicating improved tissue oxygenation due to the increase in capillarization and the reduction in FCSA, a leftward shift was observed in the superficial region. However, the hypoxia-induced adaptations in oxygen delivery (9) resulted in a slightly improved tissue oxygenation in the superficial region and a more improved tissue oxygenation in the deep region (Fig. 9C). In the deep region, mean tissue $P_O_2$ decreased from 32.1 to 5.9 mmHg after acute exposure to hypoxia. After chronic exposure, this increased to 9.9 and 18.6 mmHg (Fig. 9). In the superficial region, mean tissue $P_O_2$ decreased from 26.0 to 3.9 mmHg after acute exposure to hypoxia and decreased even further to 2.3 mmHg after taking into account the skeletal muscle adaptations to chronic exposure (Fig. 9). Use of the increased mean capillary $P_O_2$ reported by Calbet et al. (9), most likely as a consequence of the increased hematocrit and possible increase in blood flow, resulted in an improved mean tissue $P_O_2$ of 5.9 mmHg.

**Model Calculations**

Using an integrative approach to study the parameters determining oxygen delivery and utilization, we observed that the muscular adaptations to chronic hypoxia are region specific and not fiber type specific. Overall, hypoxia did cause an ~10% muscle fiber atrophy, irrespective of fiber type and muscle region. In the deep oxidative region of the plantaris muscle, this and angiogenesis resulted in improved capillarization. In the superficial glycolytic region, there was no angiogenesis but, in contrast to our expectations, a 25% increase in the oxidative capacity, indicating mitochondrial biogenesis. The absence of a change in the oxidative capacity in the deep (oxidative) region of the muscle indicates that the loss of mitochondria was proportional to the degree of atrophy. While the adaptations to chronic hypoxia in the deep region attenuated the reduction in tissue $P_O_2$ and prevented the occurrence of anoxic areas during hypoxia, the increased oxidative capacity in the superficial region appeared to aggravate problems with tissue oxygenation, which was reversed to some extent by an increase in mean capillary $P_O_2$ due to an alteration in the oxygen delivery during conditions of elevated oxygen demand by the muscle.

**DISCUSSION**

Using a model to simulate blood flow and oxygen extraction, we were able to show that adaptations to chronic hypoxia resulted in improved tissue oxygenation due to an increase in capillary density and increased capillary-to-fiber ratio (Fig. 6). Hypoxia (25 mmHg) resulted in a marked leftward shift in the $P_O_2$ distribution in the tissue; during acute hypoxia ~75% of the skeletal muscle tissue in the superficial region was anoxic and 40% of the skeletal muscle tissue in the deep region was anoxic. The adaptations of the skeletal muscle tissue to chronic hypoxia resulted in a marked region-specific adaptation: whereas in the deep region the adaptations in the skeletal muscle resulted in a rightward shift compared with the acute hypoxic situation, indicating improved tissue oxygenation due to the increase in capillarization and the reduction in FCSA, a leftward shift was observed in the superficial region. However, the hypoxia-induced adaptations in oxygen delivery (9) resulted in a slightly improved tissue oxygenation in the superficial region and a more improved tissue oxygenation in the deep region (Fig. 9C). In the deep region, mean tissue $P_O_2$ decreased from 32.1 to 5.9 mmHg after acute exposure to hypoxia. After chronic exposure, this increased to 9.9 and 18.6 mmHg (Fig. 9). In the superficial region, mean tissue $P_O_2$ decreased from 26.0 to 3.9 mmHg after acute exposure to hypoxia and decreased even further to 2.3 mmHg after taking into account the skeletal muscle adaptations to chronic exposure (Fig. 9). Use of the increased mean capillary $P_O_2$ reported by Calbet et al. (9), most likely as a consequence of the increased hematocrit and possible increase in blood flow, resulted in an improved mean tissue $P_O_2$ of 5.9 mmHg.
Relationship Between Oxidative Capacity and Capillary Supply in Normoxia and Hypoxia

The general idea is that in many species, including birds (52), humans (5), and rats (present study), the capillary supply to a fiber is related to its oxidative capacity. This relationship is modulated by the metabolic surroundings of a fiber, where a fiber in an oxidative region has a larger capillary supply than a similar fiber surrounded by glycolytic fibers (18). Here, we showed, using quantitative histochemistry, that although the number of capillaries supplying a fiber (LCFR) was indeed related to its \( V_{\text{O}_2}^{\text{max}} \), as estimated from its integrated SDH activity, LCFR correlated more with fiber size (Fig. 7) (53, 79). The lack of a correlation between SDH activity per millimeter squared of fiber and LCFR could be due, at least in part, to the fact that the mitochondrial density within a fiber decreases from the periphery to the center of the fiber (70, 79). We observed that the capillary supply per unit fiber perimeter was more or less constant [data not shown and Ref. 14], suggesting that diffusion distance or capillary contact per fiber perimeter, rather than the total number of mitochondria in a cell, is a more important determinant of the capillary supply to a fiber.

Capillaries do not only supply oxygen but also remove waste products and heat from the exercising muscle cell. This may explain the absence of a positive relationship between the integrated SDH activity and LCFR of the glycolytic fibers with a low oxidative capacity (type IIx/b fibers) in the glycolytic region of the muscle (Fig. 7B).

During exposure to hypoxia, the integrated SDH activity in these fibers increased and the relationship between spatially integrated SDH activity and LCFR became stronger for type IIx/b fibers only. This suggests that, during hypoxia, the function of the capillaries to deliver oxygen to the (previously low oxidative) muscle cells becomes more important.

Overall Adaptations to Chronic Hypoxia

Similar to previous studies, we observed no differences in the fiber type composition (30, 55). Hypoxia did, however, cause an \( \sim 10\% \) muscle atrophy (6, 20, 22, 25) in fibers of each
Region-Specific Adaptation in Oxidative Capacity

Region-Specific Increase in Capillarity

Region-Specific Adaptation in Oxidative Capacity

The spatially integrated SDH activity, reflecting the total number of mitochondria per fiber, decreased in the deep region and increased in the superficial region. In the deep region, the decreased integrated SDH activity per fiber is explicable by atrophy. This, together with an unaltered SDH activity per millimeter square, reflecting the number of mitochondria per volume of...
tissue (71), indicates that atrophy is accompanied by a proportional loss of mitochondria in the deep region. The increased integrated SDH activity in the superficial region, on the other hand, indicates that although atrophy also occurred in this region, mitochondrial biogenesis was elevated.

During hypoxia, even during submaximal exercise, an accelerated breakdown of phosphocreatine (PCr) and fall in pH occur (34), which may, independent from intracellular P2 levels, cause fatigue in single skeletal muscle fibers (68). The increased rate of accumulation of metabolites may require the earlier recruitment of glycolytic fibers during submaximal activity. This more frequent recruitment may be compared with an endurance training program inducing an increase in the oxidative capacity. An additional 5% duration of recruitment has been shown to be sufficient to increase the oxidative capacity of a muscle cell, as seen with electrical stimulation (43), and also training during hypoxia induces an increase in the oxidative capacity (1, 72). It is not clear why the oxidative capacity in the deep region is not affected, but our observations are analogous to the observation that the increase in oxidative capacity after electrical stimulation is inversely related to the original oxidative capacity of the muscle or muscle region (58).

The region-specific adaptation in mitochondrial biogenesis might be related to differential upregulation of HIF-1α in both regions as HIF-1α is reported to inhibit mitochondrial biogenesis (49). Other potential mediators for the region-specific adaptation in oxidative capacity are differences in the expression of factors that are involved in the transcriptional regulation of mitochondrial genes, such as peroxisome proliferator-activated receptor (PPAR)-γ and its ligand PPAR-γ coactivator-1α (45).

Functional Implications of the Adaptations to Hypoxia

Both the Krogh and Hill model calculations gave a qualitative indication that the improved capillarization and atrophy in the deep region of the muscle served to improve tissue oxygenation. In the superficial region, however, the adaptations did not appear to improve tissue oxygenation, as reflected by an increased proportion of anoxic tissue areas calculated with the Krogh model. The Hill model indicated that in the superficial region the PO2 allowing the muscle cell to work at VO2max was elevated after chronic hypoxia, which corresponded with an increase in the capillary PO2, calculated with the Krogh model, required for the tissue to work at VO2max without the development of anoxic tissue areas. The higher values for PO2 cap than PO2 crit (but similar qualitative changes after hypoxia) can be ascribed to the different assumptions of the models: the Hill model assumes that the PO2 around a fiber is homogeneous and diffusion thus essentially takes place from an infinite number of point sources, whereas in the Krogh model oxygen diffuses from capillaries (point sources). In the resting and isometric situations capillaries are running essentially in parallel with muscle fibers, approximating the Krogh tissue cylinders, whereas during shortening the capillaries become more tortuous, resulting in an increasing capillary-to-fiber perimeter contact and hence resembling more the Hill model (31). Nonetheless, both models agree that the increased oxidative capacity in the superficial region after chronic hypoxia does not appear to help but rather to aggravate the situation. Thus, in terms of tissue oxygenation, this seems to be an inappropriate adaptation to hypoxia.

During chronic hypoxia we did not only observed adaptations in the tissue but also an increase in hematocrit. This increase in hematocrit, together with possible increases in blood flow through the active muscle during hypoxia compared with normoxia, may explain the increased mean capillary PO2 after chronic hypoxia compared with acute hypoxia in the exercising muscle (9). When we took this into account, our model calculations showed a considerable improvement in tissue oxygenation during hypoxia, with a significant reduction in the percentage of anoxic tissue areas from 74% to 55% in the superficial region and from 19% to 6% in the deep region of the muscle (Fig. 9C).
The respiration of the mitochondria is a hyperbolic function of the PO2 that the mitochondria experience, and when the PO2 becomes too low, the respiration of the mitochondria decreases (27). Yet, the demand for ATP by the fibers in the superficial region may increase because of a more frequent recruitment, as discussed above. An increase in mitochondria may enhance the flux of oxygen and hence total respiration, even though each individual mitochondrion is not working maximally (33). According to our model calculations, there are virtually no anoxic areas during hypoxia in the deep region of the muscle, suggesting that the mitochondria may still be able to work near maximally. It is thus not necessary to increase their number to enhance flux in this region of the muscle.

Conclusions

In conclusion, using an integrative approach to study the parameters determining oxygen delivery and utilization, we observed that the adaptation of skeletal muscle to hypoxia is region specific rather than fiber type specific. Overall, hypoxia results in muscle atrophy. In oxidative regions, hypoxia results in angiogenesis, whereas fibers in a more glycolytic region of the muscle substantially increase their oxidative capacity. Without improvements in the delivery of oxygen, such as those brought about by increases in hematocrit and blood flow, the latter may cause problems with tissue oxygenation. We hypothesize that the increased oxidative capacity may serve to ensure adequate aerobic ATP generation during hypoxia by maintaining the flux of oxygen. It is currently unknown which signaling pathways underlie these region-specific adaptations to hypoxia. Finally, when considering the effects of hypoxia during the later stages of chronic obstructive pulmonary disease and chronic heart failure, where atrophy and tissue hypoxia are common observations (29), one has to bear in mind that different muscles or muscle regions might be affected differently.

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SKELETAL MUSCLE OXYGENATION IN CHRONIC HYPOXIA