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Deciphering the mysteries of myoglobin in striated muscle

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ABSTRACT

Myoglobin (Mb) is a large protein that reversibly binds oxygen in the muscle cell and is thought to be critical for O_2 supply to the mitochondria during exercise. The role of Mb in aerobic function is evaluated based on the physical properties of Mb as an O_2 carrier and experimental evidence of Mb function *in vivo*. This role depends on the reversible binding of O_2 by Mb depending on P_{O_2}, which results in: (1) storage of O_2; (2) buffering of P_{O_2} in the cell to prevent mitochondrial anoxia; and (3) parallel diffusion of O_2 (so-called, 'facilitated diffusion'). The storage role is well established in diving mammals and buffering of cell P_{O_2} above anoxic levels is shown here by *in vivo* magnetic resonance spectroscopy (MRS). However, the quantitative role of Mb in 'facilitated' or parallel diffusion of O_2 is controversial. Evidence in support of this role is from MRS analyses, which reveal rapid Mb desaturation with exercise, and from the proportionality of Mb content of a muscle to the O_2 diffusion limitation. Recent experiments with myoglobin knockout mice demonstrating high levels of aerobic function in normal and myoglobin-free mice argue against a link between Mb and oxidative phosphorylation. Thus, the current evidence supports the role of Mb in the physical diffusion of O_2; however, the unimpaired aerobic function of Mb knockout mice indicates that this role may not be critical to O_2 supply in active muscle.

Keywords body size scaling, facilitated diffusion, magnetic resonance spectroscopy, myoglobin knockout mouse, O_2 diffusion, oxidative phosphorylation.

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This review considers the role of myoglobin (Mb) in oxygen delivery in active muscle. Many excellent reviews have covered the purported mechanisms of function, mathematics of diffusion and key roles in O_2 storage, P_{O_2} buffering and facilitated diffusion of O_2 (Kreuzer 1970, Wittenberg 1970, Wittenberg & Wittenberg 1989). Despite this extensive treatment in the literature, the role of Mb in muscle function remains controversial with many papers claiming a small or non-existent role of Mb in O_2 delivery (Jürgens *et al.* 1994, Papadopoulos *et al.* 1995) and others demonstrating a relation between Mb content and aerobic function (Conley & Jones 1996). In support of its classical role in O_2 delivery is evidence that Mb-O_2 shows desaturation with exercise *in vivo* (Richardson *et al.* 1995a, Molé *et al.* 1999), which suggests that a Mb-O_2 gradient is present for Mb-mediated O_2 diffusion. The importance of this classical role would appear to be diminished by the recent finding that the myoglobinless mouse has aerobic function similar to the wild-type (Garry *et al.* 1998); however, these animals appear to have both cellular and cardiovascular adaptations that might compensate for the lack of Mb (Godecke *et al.* 1999). The goal of this paper is to provide a simple analysis of Mb's role in O_2 supply to understand how Mb functions in normal tissue *in vivo* and how muscle and cardiovascular adaptations could result in unimpaired aerobic function in Mb-knockout muscle. To achieve this goal, the authors present their work on Mb function: (1) *Mechanisms of myoglobin function* presents a simple diffusion model as a guide to Mb function in the muscle cell; (2) *Myoglobin measurements: Access to intracellular* P_{O_2} presents magnetic resonance measurements of Mb-O_2 saturation during exercise; and (3) *Myoglobinless mice* reports on studies of the myoglobin knockout mouse.

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MECHANISMS OF MYOGLOBIN FUNCTION

Paradoxes of O_2 diffusion in muscle

The first step in this analysis is to consider the role of diffusion distance in O_2 supply. Diffusion is thought to be the key pathway for O_2 supply from capillary to the respiring mitochondria and is often invoked as a limit to muscle respiratory capacity (Wagner 1993). However, several examples show no evidence for diffusion distance in limiting oxygen delivery (see Conley & Jones 1996). For example, muscles with different intercapillary distances have been found to have either similar muscle or similar mitochondria-specific metabolic rates (Hudlicka *et al.* 1988, Kayar & Weiss 1992). Thus, we are faced with the paradox that diffusion distance does not appear to significantly limit O_2 delivery or consumption *in vivo* despite the key role played by distance in diffusion.

Could parallel diffusion of O_2 and O_2-Mb be the mechanism by which the key role of distance in O_2 diffusion is minimized? Myoglobin has been shown to be an important mediator of O_2 flux (Wittenberg 1970, Wittenberg & Wittenberg 1990) and extensive modelling literature has shown how Mb-mediated diffusion could work in muscle tissue (Meyer *et al.* 1984, Popel 1989). Several results confirm this important role for Mb in O_2 supply. Mb is one of the most concentrated proteins in the cell and modelling analyses have shown that Mb content is proportional to the shortfall in O_2 supply at maximum O_2 demand (Conley & Jones 1996). Further, Mb content is very high in diving animals, indicating that the storage of O_2 afforded by a protein in mM levels provides an important source of O_2 during dives. However, there is evidence that appears inconsistent with a primary role of Mb-mediated diffusion. Mb concentration is not closely related to oxidative capacity or fibre radius as the highest Mb level is found in fibres of intermediate size and mitochondrial content (Conley & Jones 1996). In fact, the highly aerobic heart has relatively low levels of Mb [i.e. 0.25 mM (Armstrong *et al.* 1992)]. In addition, recent measurements in muscle tissue indicate that O_2 diffusivity is considerably higher and Mb diffusivity considerably lower than previously thought (Papadopoulos *et al.* 1995, Bentley & Pittman 1997). The low Mb diffusivity led Jürgens *et al.* (1994) to conclude that Mb plays a minor role in mediating O_2 flux. Clearly, an analysis of the Mb-mediated O_2 diffusion is needed to resolve these paradoxes.

A simple analysis of diffusion

Several characteristics make Mb well suited for diffusion. The equilibrium binding nature of O_2 to Mb means that binding will occur at the high P_{O_2}s characteristic of the capillary (>10 Torr) and O_2 unloading will occur at the lower P_{O_2}s in the muscle fibre (<10 Torr in active muscle). Several additional criteria necessary for Mb to significantly contribute to oxygen flux (Wittenberg 1970) have been satisfied: (1) Mb is found in concentrations sufficient for carrier-mediated diffusion (Armstrong *et al.* 1992); (2) Mb diffuses *in vivo* (Jürgens, Peters *et al.* 1994); and (3) Mb-O_2 is desaturated in exercising muscle indicating a Mb-O_2 gradient to drive Mb-mediated O_2 flux (Richardson *et al.* 1995a, Molé *et al.* 1999).

Myoglobin is a good candidate for supplementing O_2 supply because it provides a parallel pathway for diffusion in the muscle fibre. Fig. 1 shows the factors governing O_2 flux ($\dot{M}_{O_2}$) in a simple electrical circuit with conductances (inverse of resistance) through the fibre defined for O_2 (D_f) and Mb diffusion (D_{Mb}). Later we consider the full O_2 diffusion pathway from the capillary through the fibre to its core using the Fick principle (see *Myoglobin measurements: Access to intracellular* P_{O_2}), but here we consider only the fibre and examine diffusion resulting from gradients set by P_{O_2} (P_{O_2}) and Mb saturation (S_{Mb}) between the sarcolemma and fibre core:

$$\dot{M}_{O_2} = D_f(P_{O_2}) + D_{Mb}(S_{Mb}) \quad (1)$$

The conductances reflect the fibre size (r^{-2}), content (oxygen solubility, α_{O_2} or myoglobin concentration, [Mb]) and the diffusivities of O_2 (D_{O_2}) and Mb (D_{Mb}) for diffusion into a cylinder:

$$D_f = 4 \cdot \alpha O_2 \cdot DO_2 \cdot r^{-2} \quad (2)$$

$$M_{O_2} = (\Delta P_{O_2})\, D_f + (\Delta S_{Mb\text{-}O_2})\, D_{Mb}$$

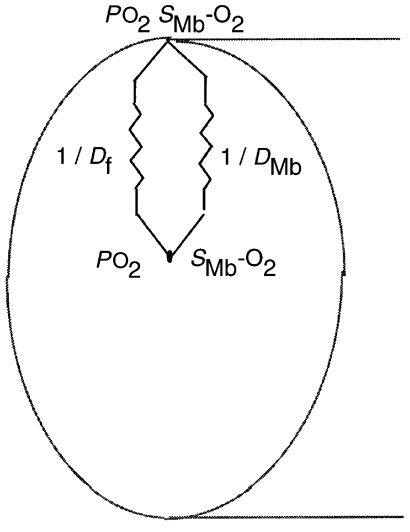

Figure 1 Diagram of a simple diffusion model of O_2 flux in muscle fibres.

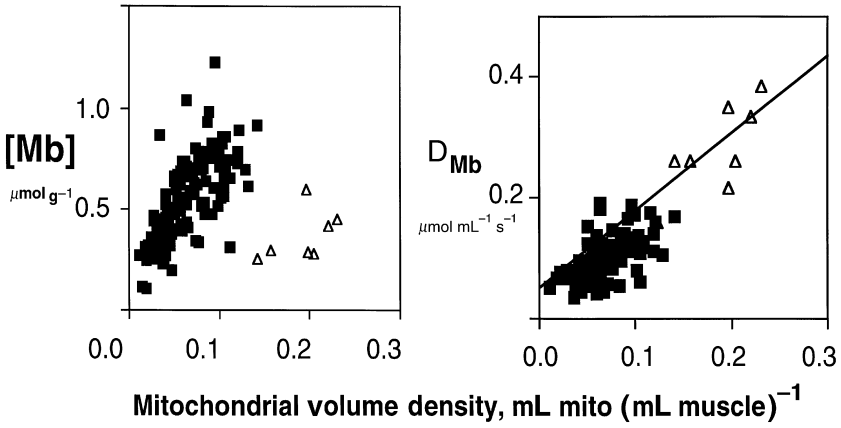

Figure 2 Myoglobin concentration ([Mb]) and diffusion conductance (D_{Mb}) as a function of mitochondrial volume density of horse and steer muscle. ■, From skeletal muscle; △, from the heart. The original data are reported in Armstrong *et al.* (1992) and Conley & Jones (1996).

$$D_{Mb} = 4 \cdot [Mb] \cdot D_{Mb} \cdot r^{-2} \quad (3)$$

Thus, Mb provides a parallel pathway for O_2 diffusion driven by a separate gradient (i.e Mb-O_2 gradient).

High Mb content trades-off for low diffusivity

The large size of the Mb molecule (17 500 mw) compared with O_2 would appear to make Mb a poor candidate for parallel diffusion of O_2. The 100-fold difference in the diffusivity of O_2 and Mb would suggest that Mb-mediated O_2 flux would make a trivial contribution to O_2 supply. However, the tissue conductances depend both on the diffusivities and tissue concentrations of O_2 and Mb as defined in Eqns 2 and 3. The tissue concentrations of these molecules are also nearly 100-fold different, but in the opposite direction, with the low solubility of O_2 in tissues resulting in [O_2] in the μM range compare to typical [Mb] in the mM range. Thus, the slow diffusivity of the Mb molecule is compensated for by large tissue concentrations that result in a similar conductance (D_{Mb}) as compared with O_2 alone (D_f).

Myoglobin conductance, not concentration, is important

Mb content alone is a poor predictor of its role in O_2 delivery as illustrated in Fig. 2, which compares [Mb] with the mitochondrial volume density ($V_{V(mt,f)}$) of muscle samples from horses and steers. The heart has the highest $V_{V(mt,f)}$ and therefore maximal O_2 demand, yet this tissue has [Mb] less than half the level found in skeletal muscles with a third the $V_{V(mt,f)}$. Equation 3 shows that concentration alone does not determine the conductance to Mb, but that fibre radius is also a critical factor. Figure 2 also shows that the combination of fibre radius and Mb content contained in the Mb diffusion conductance results in a continuous relation of D_{Mb} with $V_{V(mt,f)}$. Thus, the two factors critical to O_2 diffusion in the fibre – fibre radius and [Mb] – in combination result in a diffusion conductance that is closely correlated with mitochondrial content and therefore O_2 flux.

These conductances represent only the capacity for O_2 supply while the actual flux via O_2 and Mb-O_2 diffusion depends on the P_{O_2} levels and gradients in the muscle. A simple diffusion model in the fibre (Eqn 1) is evaluated for these O_2 fluxes and conductances. This simple model permits determination of how much of this potential of Mb-mediated diffusion is used in O_2 delivery. Figure 3 shows the fraction of O_2 carried by Mb vs. free diffusion as a function of [Mb] for three ratios of the diffusivities for Mb (D_{Mb}) to O_2 (D_{O_2}). Clearly, a larger fraction of the O_2 flux is carried by Mb as its concentration in the cell increases. Also, a larger fraction is carried by Mb-O_2 as diffusivity of Mb relative to O_2 increases. The value of both diffusivities have recently changed as new techniques have emerged to better measure this property. Recently, the D_{O_2} value was revised upward by directly measuring the value at tissue temperatures expected in exercising muscle (i.e. 40 °C) (Bentley & Pittman 1997). The D_{Mb} value was revised downwards based on *in situ* spectroscopic measurements at physiological temperature (Jürgens *et al.* 1994, Papadopoulos *et al.* 1995), but measurements by magnetic resonance in intact, beating cardiac muscle support the higher D_{Mb} values (Wang *et al.* 1997). Direct measurements of the quantitative role of Mb in O_2 delivery are needed to resolve this discrepancy in D_{Mb} and determine which Mb diffusivity value governs Mb-mediated O_2 diffusion.

Mb content vs. diffusion limitation

Demonstration of a diffusion limitation to O_2 supply would indicate that additional O_2 delivery via Mb is needed to meet the muscle O_2 demand. We previously reported an analysis of O_2 delivery in muscle tissue in

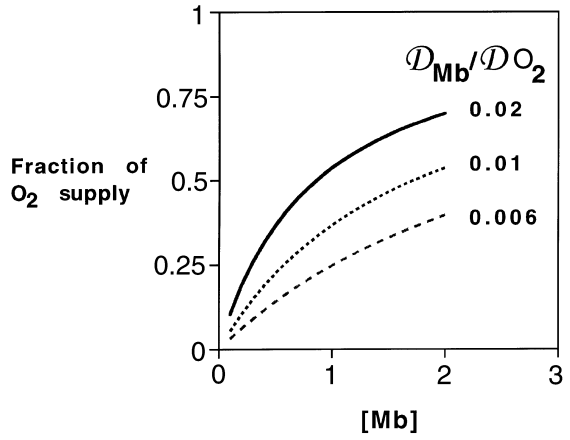

Figure 3 Myoglobin contribution to O_2 supply as a function of myoglobin concentration and the ratio of the myoglobin to oxygen diffusivity (D_{Mb}/D_{O_2}).

horse and steers running at rates that evoke the maximum oxygen uptake (Conley & Jones 1996). Our analysis found that many fibres had an O_2 demand that exceeded the capacity for O_2 supply by diffusion as the muscle reached its maximum oxygen uptake. The lowest Mb content was found in those fibres without a diffusion limitation (O_2 demand/O_2 diffusion = 1) and was highest in the fibres with the greatest diffusion limitation (O_2 demand/O_2 diffusion > 1). These findings indicated that Mb was needed as a parallel source of O_2 supply and that the Mb content increased in proportion to the size of the diffusion limitation to mitochondrial O_2 demands.

Mb scaling: Paradox of body size on diffusion and oxidative capacity

Nature has provided us with a good experiment to test the role of myoglobin in O_2 delivery using the scaling of muscle and physiological properties with body size. The classic mouse-to-elephant curve illustrates the point that resting O_2 uptake increases per body mass with decreasing size. A similar increase per muscle mass is seen in the maximum O_2 uptake (Seeherman *et al.* 1981) and muscle mitochondrial volume density (Mathieu *et al.* 1981). Thus the maximum O_2 flux per muscle mass is many-fold higher in a 0.05 kg mouse as compared with a 500-kg horse. If myoglobin content were proportional to total flux, the content would be expected to increase as the maximum flux per mass rises (and body mass decreases). Instead, the opposite is apparent in Fig. 4 where Mb content is shown to increase with body mass in the Psoas muscle in animals ranging in size from the pigeon to the blue whale (Lawrie 1953). Elimination of the one diving mammal – the blue whale – has little effect on the strong relationship between Mb content and body mass shown in this figure.

How do we explain the increase in Mb content in the face of reduced maximal O_2 demand? The answer lies in the changing muscle fibre and cardiovascular properties with body size. First, the driving force for O_2 delivery from the blood increases as body size decreases. More than 40 years ago, the P_{50} of blood was found to increase with lower body size (Schmidt-Neilsen & Larimer 1958) and, more recently, this P_{50} scaling was found to result in a higher mixed venous P_{O_2} in smaller animals exercising at $\dot{V}_{O_{2max}}$ (Kaya *et al.* 1994). Thus, smaller mammals have a larger P_{O_2} driving O_2 diffusion into the muscle cell, especially at the end of the capillary where the magnitude of the gradient is critical to O_2 and Mb-mediated diffusion.

At the same time that the O_2 pressure head is rising, fibre diameter is decreasing and capillary density is increasing with smaller body size (Hoppeler *et al.* 1981). The consequence of these changes is a large increase in O_2 conductance (Eqn 2) as well as a decrease in intercapillary distances. The result is that smaller mammals not only have a larger pressure head for O_2 diffusion into the muscle cell but an increased conductance for O_2 diffusion as well. Thus, one way to explain the decrease in Mb content with decreasing body size is by the reduced need for supplemental O_2 supply because of an increased O_2 diffusion capacity in smaller vs. larger animals.

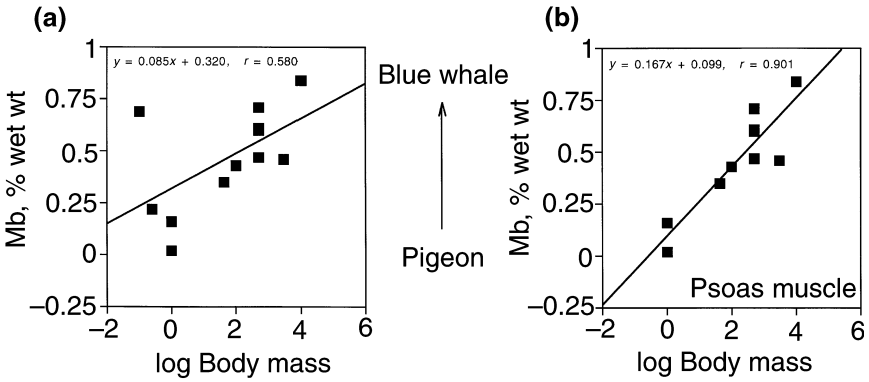

Figure 4 Scaling of myoglobin content (Mb content) in muscles as a function of body mass. (a) [Mb] in a variety of cardiac and skeletal muscles from animals ranging from pigeons to the blue whale. (b) The scaling of Mb content in a single muscle (psoas) in mammals with body mass. Data are taken from Lawrie (1953).

The two examples reported here not only demonstrate the important role of Mb in O_2 supply, but also the interaction of muscle properties in the balance of O_2 supply to O_2 demand. First, Mb content was found to be higher in fibres with an O_2 diffusion limitation in horse and steer muscle exercising at the maximum oxygen consumption (Conley & Jones 1996). Second, lower Mb content in smaller animals accompanied the reduction in the O_2 diffusion limitation resulting from changes in cellular and cardiovascular properties with body size. Key to this modelling is that Mb-O_2 desaturates during exercise and generates a gradient in the muscle fibre down which parallel Mb-O_2 diffusion can occur. In the following section, Richardson reports *in vivo* magnetic resonance measurements of Mb-O_2 saturation levels in exercising human limb muscle. The final section considers life without myoglobin. Could the reduced O_2 diffusion limitation in small mammals and adaptations that balance O_2 supply to O_2 demand permit normal aerobic function in the absence of myoglobin? The aerobic properties and adaptations of the myoglobin knockout mouse presented in the final section will help to answer this question.

MYOGLOBIN MEASUREMENTS: ACCESS TO INTRACELLULAR P_{O_2}

The recent ability to detect myoglobin (Mb) desaturation utilizing proton magnetic resonance imaging (MRS) has made it possible to measure intracellular P_{O_2}, the final and somewhat elusive last step in the oxygen cascade from air to muscle (Wang *et al.* 1990, Noyszewski *et al.* 1997). Proton MRS, unlike most previous techniques that address tissue oxygenation, is non-invasive, is without deleterious effects, and therefore very well suited for *in vivo* human studies (Thomas & Morris 1981). The functional isolation of the quadriceps muscle group has been a useful model to study exercising muscle in man (Andersen *et al.* 1985, Richardson *et al.* 1993, Radergran 1997) and allows the measurement of effluent (femoral) venous P_{O_2} and the calculation of mean capillary P_{O_2} (Richardson *et al.* 1995b). In combination, this proton MRS technique to determine Mb saturation and the functionally isolated human quadriceps muscle model (Andersen *et al.* 1985, Richardson *et al.* 1995a, b, Richardson *et al.* 1998) have provided the opportunity to study the relationships between intracellular and intravascular events in humans during exercise.

Rapid Mb desaturation at submaximal exercise

The maximum deoxy-Mb signal is apparent even at 50% of $\dot{V}O_{2max}$ (Fig. 5) and this desaturation occurs very rapidly. There is a rapid disappearance of the deoxy-Mb signal (re-saturation) when exercise is ceased for less than 45 s, for instance to adjust a subject's shin brace during knee-extensor exercise. This rapid Mb desaturation to 50–60% is indicative of the immediate use of at least half of the Mb O_2 stores. Thus, the speed and magnitude of this response may have several functions. First, the immediate availability of 50% of the stored Mb-associated O_2 may be an important O_2 source for the increased oxidative metabolism at the start of exercise. Second, this Mb desaturation reduces the carrier-depleted region, maximizing the P_{O_2} gradient from blood to cell (Fig. 6). Consequently, the passive transport system responsible for O_2 influx into the muscle cell is facilitated by this rapid desaturation, even during light exercise. The greater deoxy-Mb signal in hypoxia (Fig. 5) and the concurrent elevation in the O_2 diffusional conductance in hypoxia throughout submaximal exercise (Fig. 7), supports these concepts. The effective diffusivity at submaximal WR's in hypoxia was markedly elevated in comparison with normoxic values. At ≈65% of $\dot{V}O_{2max}$ the diffusional conductance in hypoxia was already 85% of the maximum recorded value, whereas in normoxia at a comparable $\dot{V}O_2$ conductance was only 59% of the maximum value. This difference may be the result of the increased concentration of available O_2 carrier within the muscle tissue (deoxy-Mb) (Honig & Gayeski 1993) (Fig. 7).

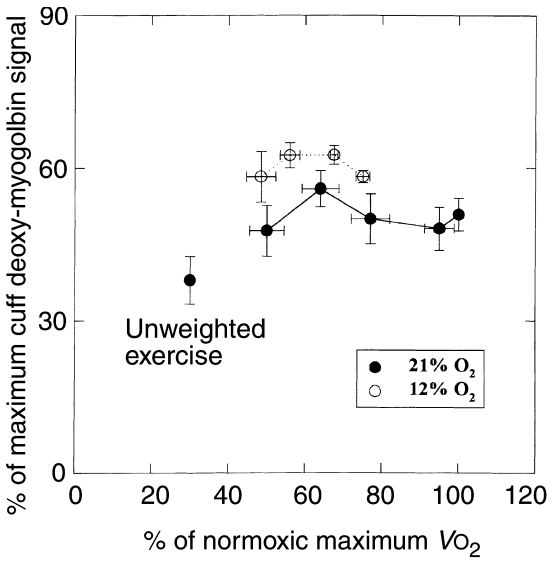

Figure 5 The percentage of maximum cuff deoxy-Mb signal in hypoxia and normoxia as a function of the percentage of maximum normoxic leg $\dot{V}O_2$ during knee-extensor exercise.

Blood to cell P_{O_2} gradients

The use of proton MRS and the knee-extensor model have provided the first *in vivo* experimental evidence of

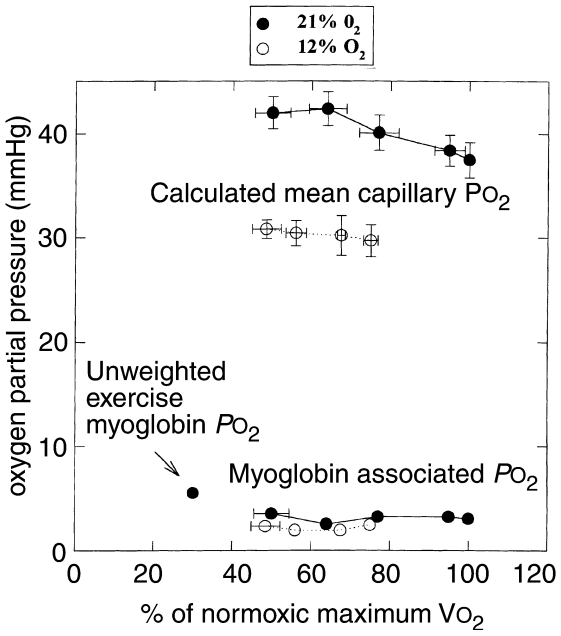

Figure 6 Calculated mean capillary Po_2 and cellular Mb-associated Po_2 in relation to the percentage of normoxic Vo_2 in both normoxia and hypoxia. Note the large difference between the Po_2 available in the capillaries and the Po_2 at the cellular level, even at submaximal work rates.

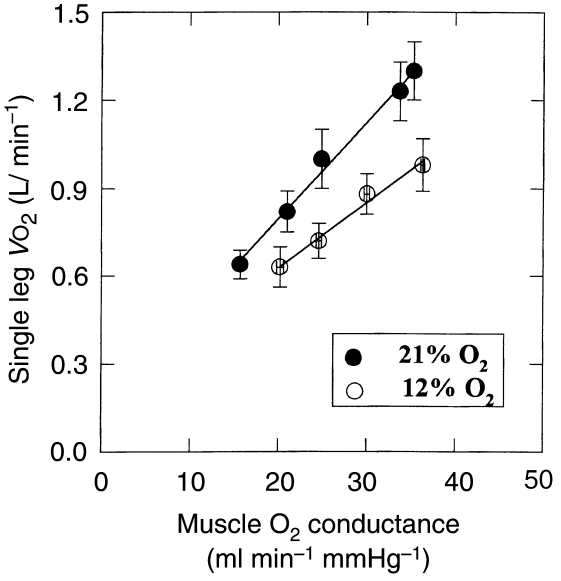

Figure 7 The relationship between Vo_2 and muscle O_2 conductance. Until the measurement of Mb-associated Po_2 was achieved this analysis of diffusivity was only possible at Vo_{2max}, where mitochondrial Po_2 was assumed to be very close to zero and hence could be ignored.

a steep O_2 gradient from blood to intracellular sites in human skeletal muscle *in vivo*. These independent numerical estimates reveal that O_2 pressure in the blood during exercise was 11–12-fold higher than in the cytoplasm at submaximal WR's (50% leg Vo_{2max}) and remained 8–10-fold higher even at WR_{MAX}. This equates to a Po_2 gradient from mean capillary blood to Mb of 39 (normoxia, N) and 29 mmHg (hypoxia, H) at submaximal WR's, which fell slightly to 35 (N) and 27 mmHg (H) at WR_{MAX}.

Constant Mb desaturation despite progressively intense exercise

Both intuition and theoretical modelling (Severinghaus 1994) suggest it is reasonable to expect intracellular Po_2 to fall as the intensity of exercise increases to enhance the O_2 flux into the muscle cell. Repeated investigations utilizing the isolated knee-extensor model have reported an increase in Mb desaturation from rest to exercise, but have not documented a progressive fall in intracellular Po_2 from moderate to maximum exercise (Richardson *et al.* 1995b, Richardson *et al.* 1998) (Figs 5 and 6). However, as Fick's law of diffusion illustrates [$Vo_2 = Do_2$ ($P_{cap}O_2 - P_{mito}O_2$], Do_2 also regulates O_2 flux. A major determinant of Do_2 is the effective surface area available for diffusion (Federspiel & Popel 1986). If Do_2 increases in response to capillary recruitment and increased dynamic haematocrit (Gorzynski & Duling 1978, Lindbom *et al.* 1980, Honig *et al.* 1982), Mb-associated Po_2 need not change with increasing exercise intensity. In fact, consistent with this interpretation, the calculated Do_2 for the present data did increase with each increased WR (Fig. 7) thus, indicating that O_2 conductance is recruited proportionally as Vo_2 increases, but $P_{Mb}O_2$ remains constant after an initial fall during exercise eliciting submaximal Vo_2.

Data that refute this interpretation have recently been published (Molé *et al.* 1999), indicating that, during progressive plantar flexion exercise, Mb appears to desaturate linearly with increasing work rate. Using these data, the authors conclude that Do_2 remains constant and it is in fact the gradient from blood to cell that increases allowing a greater O_2 flux. However, there are several important distinctions that need to be recognized when comparing MRS data collected in two exercise paradigms as different in physiological response as knee-extensor exercise and plantar flexion exercise (Richardson 1999). For example, at maximal exercise Molé *et al.* (1999) reported a pH indicative of a [H^+] of 135 nEq L^{-1} vs. knee-extensor study values of 269 nEq L^{-1} at maximal exercise. This is undoubtedly a result of the muscle groups studied and not NMR methodology as we too have found similar pH values in the gastrocnemius during maximal effort of around 6.9 (Hogan *et al.* 1999), while we have recently again recorded pH values of 6.47 ± 0.16 in untrained subjects during maximal knee-extensor exercise. With the size of surface coil used by Molé *et al.* (1999) (5 in. diameter), it is most likely that a significant

contribution to the signal was attained from the soleus as well as the gastrocnemius (both myoglobin and phosphorus). In this regard, it has previously been demonstrated that line width for the P_I, which reflects relative homogeneity of the pH values within the tissue being observed, can alter significantly during progressive plantar flexion exercise indicating functional heterogeneity (Barstow *et al.* 1994). This heterogeneity has previously been reported as an important limitation in the interpretation of MR data in the calf muscle (Vanderbourne *et al.* 1991). This potential overlap into a muscle rich in slow-twitch fibres (soleus) is additionally supported by the observation that at maximum work levels the percentage phosphagen shifts reported by Molé *et al.* (1999) were the same as previously observed at only 40–50% of aerobic maximum (Richardson *et al.* 1995b, Allen *et al.* 1997).

Intracellular P_{O_2} as a determinant of $\dot{V}O_{2max}$

Intracellular P_{O_2} measurements have contrasted the linear relationship between mean capillary and intracellular P_{O_2} and the hyperbolic relationship between intracellular P_{O_2} and $\dot{V}O_{2max}$ (Richardson *et al.* 1999) (Fig. 8). This suggests that in hyperoxia there is the expected rise in intracellular P_{O_2} (owing to increased mean capillary P_{O_2}), but this elevated O_2 availability is now in excess of mitochondrial capacity (Fig. 8). Evidence that intracellular P_{O_2} is a determinant of $\dot{V}O_{2max}$ when F_iO_2 is manipulated from 0.12 to 0.21 and 1.0. However, in the latter case the increased intracellular

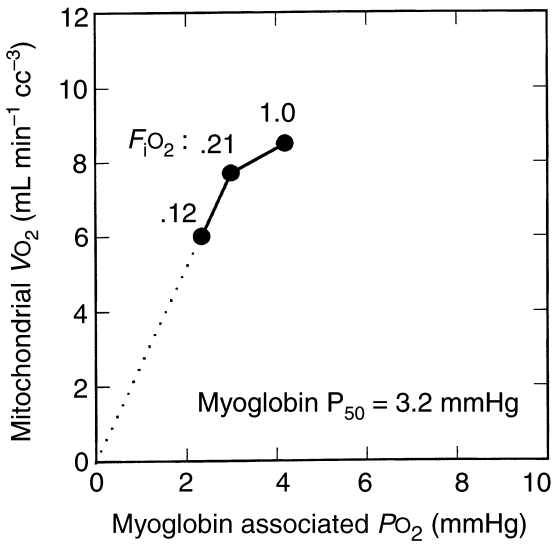

Figure 8 *In vivo* measurements of the relationship between mitochondrial O_2 uptake and intracellular P_{O_2}. Mitochondrial $\dot{V}O_2$ was calculated based upon an assumed mitochondrial fibre volume of 7.5%, myofibril volume of 80%, and muscle density of 1.06 g cc^{-1}. Muscle mass was 2.5 kg, calculated from anthropometric measurements.

P_{O_2} appears to result in diminishing returns with respect to an increase in $\dot{V}O_{2max}$. These observations are consistent with cellular metabolism that is moving toward a transition between O_2 supply as a determinant of $\dot{V}O_{2max}$ and O_2 demand as a determinant of $\dot{V}O_{2max}$. Further increases in intracellular P_{O_2}, beyond those recorded in hyperoxia, would be anticipated to have smaller effects upon $\dot{V}O_{2max}$ until a plateau is reached and $\dot{V}O_{2max}$ becomes invariant with intracellular P_{O_2}. From this point intracellular P_{O_2} is no longer a determinant of skeletal muscle $\dot{V}O_{2max}$. This hyperbolic relationship, originating from the origin, between O_2 tension and cellular respiration is in agreement with data previously described by Wilson *et al.* (1977) in kidney cells. We again suggest (Richardson *et al.* 1995a), although now with more conclusive data, that these findings may represent the hyperbolic relationship between *in vivo* muscle $\dot{V}O_2$ and intracellular P_{O_2}, supporting the concept that maximal respiratory rate ($\dot{V}O_{2max}$) in trained skeletal muscle is normally limited by O_2 supply.

It is also important to recognize that although the magnitude of the intracellular P_{O_2} changes reported here are small, they appear to have biological significance, based upon the observation that $\dot{V}O_{2max}$ fell 35% from hyperoxia to hypoxia (Fig. 8). This raises the issue of the critical P_{O_2} ($P_{O_{2crit}}$), below which maximal mitochondrial rate is compromised. Previously, using Mb cryomicrospectroscopy in dog gracilis muscle, Connett *et al.* (1983, 1984, 1986) were unable to find loci with a P_{O_2} of less than 2 mmHg, but elevated blood lactate levels in the muscle effluent. As previous investigations (Chance & Quistorff 1978) suggested that $P_{O_{2crit}}$ may be between 0.1 and 0.5 mmHg, Connett *et al.* (1984) concluded that elevated blood lactate concentrations must be caused by factors other than simply O_2-limited mitochondrial ATP synthesis rate. We have recently supported this conclusion by providing *in vivo* data in man indicating that average intracellular P_{O_2} remains above these values even at maximal exercise in hypoxia, with rapidly rising lactate production (Richardson *et al.* 1998a) (Fig. 9). With the recognition that muscle lactate production may not be the result of cellular hypoxia (Richardson *et al.* 1998a), the present data are suggestive of a much higher $P_{O_{2crit}}$ *in vivo* in exercise trained human skeletal muscle as maximal mitochondrial metabolic rate appears to be significantly compromised when intracellular P_{O_2} falls from a level around 4 mmHg (Fig. 8)

Intracellular P_{O_2} vs. mitochondrial P_{O_2}

At least two thought provoking questions are raised based upon the recent assessment of intracellular P_{O_2} using Mb desaturation: (1) Why at normoxic or

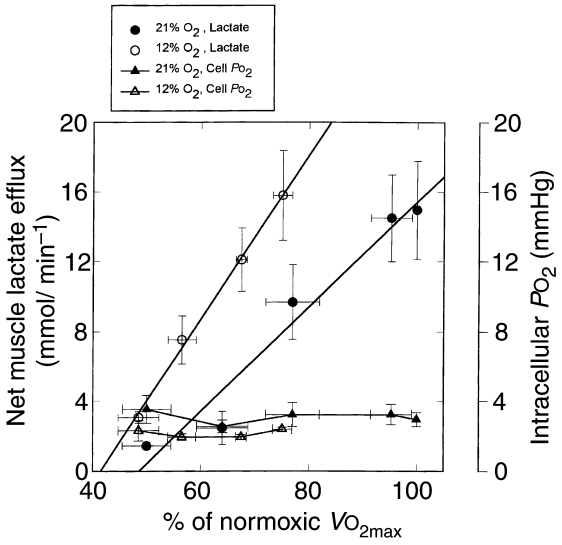

Figure 9 Net muscle lactate efflux and intracellular P_{O_2} as a function of O_2 consumption in normoxia and hypoxia.

hyperoxic $V_{O_{2max}}$ did the Mb-associated P_{O_2} not fall to the level reached in hypoxia, and (2) why in each of the three varied F_{iO_2} was the Mb desaturation far less at maximum exercise than under conditions of cuff occlusion? A possible explanation may be found by attempting to reconcile our Mb-associated data with the recent measurements of cytochrome a,a_3 oxidation–reduction state in exercising skeletal muscle (Duhaylongsod *et al.* 1993). These data illustrated a progressive decrease in the concentration of oxidized cytochrome a,a_3 (which correlated highly with rising muscle lactate efflux), with increasing muscle O_2 extraction. At $V_{O_{2max}}$ the magnitude of this redox response was equivalent to that observed at death or complete anoxia, suggesting that near depletion of O_2 at the mitochondrial level accompanies maximal exercise intensities (Duhaylongsod *et al.* 1993). These findings are in stark contrast to our Mb-associated P_{O_2} data which revealed a constant intracellular P_{O_2} (which did not correlate with rising muscle lactate efflux (Richardson *et al.* 1998) (Fig. 9), with increasing work intensity. However, a reconciliation of these data is possible by approaching them with similar logic employed to explain the observation that there is a large gradient from blood to cell and venous P_{O_2} (representative of end capillary P_{O_2}), does not fall to zero even at $V_{O_{2max}}$ (Wagner *et al.* 1990, Richardson *et al.* 1995b): The concept that there is a finite O_2 conductance (D_{O_2}) and that this may limit O_2 transport (Wagner 1992). Thus a mitochondrial D_{O_2}, which limits O_2 conductance from the cytosol to mitochondria, may explain both the difference in oxygen availability outside and within the mitochondria as well as the inability for Mb-P_{O_2} to fall to a greater extent before the cessation of high intensity exercise. In this scenario, a gradient exists from capillary to cytosol (≈30 mmHg) and from cytosol to mitochondria (≈2 mmHg). It should be noted that although the gradient is vastly different in each case the physiological significance may well be of equal importance.

Lactate production and intracellular oxygen availability

There is considerable circumstantial evidence to support the notion that lactate production is related to inadequate O_2 availability during exercise (Wasserman 1984, Katz & Sahlin 1988). However, recent ^{31}P MRS demonstrate no difference in glycolytic flux, and therefore lactate production, in exercising human muscle under normoxic vs. ischaemic conditions (Conley *et al.* 1998). This result and the recent use of proton MRS to detect Mb desaturation indicates that lactate generation occurs under fully aerobic conditions (Richardson *et al.* 1998a). Earlier evidence in support of aerobic lactate generation (Jobsis & Stainsby 1968) is the finding of the similar oxidation of NADH/NAD^+ at rest and in lactate-producing muscle. One would expect a reduction in the members of the respiratory chain (including the NADH/NAD^+ pair) to coincide with increased lactate production if lactate output was caused by O_2-limited oxidative phosphorylation. With another approach, Mb cryomicrospectroscopy in dog gracilis muscle, Connett *et al.* (1983, 1984, 1986) were unable to find loci with a P_{O_2} of less than 2 mmHg. As previous investigations (Chance & Quistorff 1978) suggested that the critical P_{O_2} ($P_{O_{2crit}}$), below which maximal mitochondrial rate is compromised (between 0.1 and 0.5 mmHg), Connett *et al.* (1984) concluded the elevated La concentration must be caused by factors other than simply O_2-limited mitochondrial ATP synthesis rate. Our Mb-associated P_{O_2} data support and extend these latter observations by providing *in vivo* data in man and suggest that average intracellular P_{O_2} remains above these previously reported values for $P_{O_{2crit}}$ even at maximal exercise in hypoxia (Richardson *et al.* 1998).

With respect to the concept of the 'anaerobic' threshold these data demonstrate that during incremental exercise skeletal muscle cells do not become 'anaerobic' as lactate levels suddenly rise, because intracellular P_{O_2} is well preserved at a constant level, even at maximal exercise (Fig. 9). Thus our data illustrate the lack of a relationship between intracellular P_{O_2}, lactate efflux and muscle pH. However, the observation that in hypoxia intracellular P_{O_2} and muscle $V_{O_{2max}}$ are reduced and muscle lactate efflux is accelerated leaves open the possibility that intracellular P_{O_2} may still play a role in modulating muscle metabolism and ultimately muscle fatigue.

MYOGLOBINLESS MICE

Transgenic technologies have provided scientists with unique opportunities to study the effects of gain-of-function or loss-of-function on animal development and performance. In the case of the former, the introduction of a transgene that results in the over-expression of a particular protein can provide important information not only about the normal function of that protein, but also about its potential role in the development of or protection against a particular disease process. As an example, over-expression of the inducible 70-kDa heat shock or stress protein, HSP72, in transgenic mice has been shown to have a cardioprotective effect against ischaemia and reperfusion injury (Radford *et al.* 1996). Likewise, eliminating a protein through gene targeting or knockout techniques can provide valuable information about the predicted role of the protein, as well as unexpected findings of unanticipated functions. For instance, a recent study by Chemelli *et al.* (1999) found that targeted disruption of the *orexin* gene, originally being studied for its effects on energy homeostasis, resulted in a phenotype with marked similarity to that of human patients with narcolepsy. Thus, although the results of studies of transgenic or knockout animals are often 'predictable' or confirmatory in nature, many others have been surprising, providing valuable insights into mechanisms regulating normal and abnormal physiology. Recent reports describing the effects of the loss of myoglobin in mice are examples of the latter (Garry *et al.* 1998, Godecke *et al.* 1999). They present intriguing findings that depict the apparent lack of effect of the loss of this haemoprotein, as well as potential adaptive responses that provide clues regarding the role of myoglobin in normal muscle function.

No apparent phenotype for myoglobin knockouts

'Myoglobinless' mice were created using targeting strategies that deleted exon 2 of the myoglobin gene (Garry *et al.* 1998, Godecke *et al.* 1999). The deleted region encodes nearly one-half of the 154 amino acids that comprise the myoglobin protein, including the essential heme-binding domain. Disruption of the myoglobin gene was confirmed by Southern blot analysis of digested genomic DNA and by the absence of RNA transcripts that included exon 2 as detected by reverse-transcription/polymerase chain reaction assay. In addition, myoglobin protein could not be detected in cardiac or skeletal muscles of myoglobin-deficient mice either by immunohistochemical assays or by Western analysis. Further, the muscles of the myoglobin-deficient mice were pale, reflecting the complete lack of pigmentation normally provided by this protein.

Following birth, myoglobinless mice had no apparent phenotype (Garry *et al.* 1998, Godecke *et al.* 1999). They grew normally, and reached sexual maturity and were fertile at an age comparable with that of their wild-type and heterozygous littermates. Myoglobin knockout mice displayed no obvious signs of cardiac or skeletal muscle dysfunction, and survived at rates similar to those for normal mice.

Stressing the systems

The question then arose as to whether myoglobinless mice would be able to meet the demands of stresses involving a need for increased oxygen transport. To address this, myoglobin-deficient and wild-type mice were evaluated in a facility in the Department of Molecular and Cellular Physiology at the University of Cincinnati that was designed especially for the testing of transgenic mice (Garry *et al.* 1998). The mice were subjected to two protocols, one involving exercise performance on a motor-driven treadmill and the other a challenge to a hypoxic gas mixture. In both cases, the myoglobin knockout mice responded in a manner that was indistinguishable from their wild-type counterparts. Endurance performance at high levels of exercise intensity was identical between the two groups, as were the ventilatory responses to the hypoxic challenge (13.5% O_2).

Studies of isolated cardiac and skeletal muscle function yielded similar results. Hearts isolated from myoglobin-deficient mice displayed similar indices of function as those from wild-type mice when challenged with increases in preload (Garry *et al.* 1998) or increasing doses of dobutamine (Godecke *et al.* 1999 and unpublished results). Likewise, soleus and extensor digitorum longus skeletal muscles isolated from myoglobin knockout and wild-type mice had identical patterns of fatigue (Garry *et al.* 1998). Interestingly, the similarity of responses for both cardiac and skeletal muscle was independent of the degree of oxygenation of the solutions perfusing or bathing the isolated hearts or muscles (Garry *et al.* 1998).

Some adaptations and explanations

Given the characteristics and regulation of myoglobin described in the first section of this paper, these results from myoglobinless mice appear to hinder rather than enable a deciphering of the mysteries of this protein. The surprising and perhaps unexpected results seem to indicate that myoglobin plays little if any role in normal cardiac and skeletal muscle function. Alternatively, there may be subtle adaptive mechanisms that promote and permit viability and normal function in the absence of myoglobin. Supporting the

latter, there also were a number of adaptive changes noted that might help explain the apparent lack of effect of eliminating this O_2-binding protein. First, myoglobin-deficient mice were reported to have increased haemoglobin levels (Godecke *et al.* 1999). Although modest, the altered haemoglobin levels would increase the arterial O_2 carrying capacity by ≈8%. Second, the myoglobin knockout mice appeared to have increased myocardial capillary density (Godecke *et al.* 1999). This increase in capillary density would thus reduce the O_2 diffusion distance between red blood cell and muscle fibre membrane. Third, the myoglobin-deficient mice were reported to have increased coronary flow and increased coronary flow reserve compared with their wild-type counterparts (Godecke *et al.* 1999). This would result in an increase in overall blood flow and O_2 delivery both at rest and during periods of elevated cardiac function. Taken together, these adaptive changes would increase the P_{O_2} gradient from red blood cell to mitochondrion and thus perhaps offset the absence of any intracellular myoglobin-mediated O_2 diffusion. In addition, microarray analysis of cardiac muscle from myoglobin null and wild-type mice showed dysregulation of gene expression supporting a molecular adaptive process in mice that lack myoglobin (D. Garry, unpublished results).

There are a number of possible conclusions that might be drawn from the findings of these studies of myoglobinless mice. One, which is based on the results of the exercise and isolated heart and skeletal muscle studies, is that myoglobin plays little or no role in normal cardiac and skeletal muscle function. Clearly, the intact knockout mice, as well as the hearts and skeletal muscles isolated from them, responded robustly to the stresses imposed upon them and were able to function normally. A second possibility is that the knockout mice in these studies were not stressed sufficiently to see the effects of a lack of myoglobin. Studies in which myoglobin-deficient mice are exposed acutely or chronically to extreme altitude and/or are exercised at altitude would test this hypothesis. However, the fact that isolated heart and skeletal muscle function was identical between knockout and wild-type mice both in the presence and absence of O_2 provides strong evidence against this possibility. A third conclusion is that the findings are unique to mice, a small mammal endowed with relatively low levels of myoglobin (see *Mechanisms of myoglobin function*). This is not unique to the myoglobin protein, but rather is a concern for the vast majority of transgenic and knockout experiments in which mice are the animal model. Very simply, mice may not be a good model for the study of O_2 transport. Studies of larger mammals with greater O_2 diffusion distances and higher levels of myoglobin might reveal impairment in exercise capacity or in cardiac or skeletal muscle function in the absence of this protein. Finally, the apparent adaptive responses displayed by the myoglobin-deficient mice allow these animals to function normally, and demonstrate the remarkable ability of organisms to compensate for disruptions in normal structure or function. The tissue, cellular, and molecular differences already noted between the myoglobin null and wild-type mice indicate that, although perhaps not necessary for normal muscle function, this conserved haemoprotein may contribute to O_2 delivery and/or storage. Equally importantly, myoglobin may have other essential functions such as serving as a buffer for oxygen-derived free radicals or nitric oxide.

The value of transgenic and knockout animals

Clearly, transgenic technologies will not provide answers to all fundamental questions in biology. What they do provide, however, is a powerful tool with which scientists can begin to address important biological questions within an integrative framework. Transgenic and knockout animals are valuable models that can be used to answer new and old questions alike about O_2 delivery and utilization. In addition, they allow scientists opportunities to re-examine previous studies and experiments in a new setting. For example, earlier studies using carbon monoxide or chemical reagents (Wittenberg & Wittenberg 1975, Doeller & Wittenberg 1990) that showed an important functional role for myoglobin might be revisited using myoglobin-deficient mice. Regardless of which of the above conclusion(s) concerning myoglobinless mice is correct, these unique animals have provided new insights and impetus to our studies of O_2 transport.

CONCLUSIONS

A simple diffusion model provides insight into the role of Mb in intracellular O_2 supply. First, the need for parallel diffusion by O_2 and Mb-O_2 was demonstrated by showing that muscle fibres with a diffusion limitation to O_2 supply typically have higher Mb content than do fibres with no O_2 diffusion limitation. Thus, the higher capillary P_{O_2}, smaller muscle fibre size, and larger capillary density in small vs. large mammals results in a greater capacity for O_2 diffusion alone with less need for parallel diffusion by Mb. Direct measurement of Mb saturation by magnetic resonance demonstrates that Mb-O_2 does desaturate with exercise. These results show that the cellular P_{O_2} is low enough for unloading of O_2 from Mb-O_2 and therefore that parallel diffusion of O_2 is possible. Further, these MRS results show that, on average, the muscle does not

become anoxic even at exercise levels eliciting maximum oxygen consumption. Thus, the lactate generation characteristic of exercise at the aerobic maximum does not reflect cellular anoxia, but rather a higher rate of glycolytic flux than is needed to sustain oxidative phosphorylation. Finally, the maintenance of aerobic function in the myoglobin knockout mouse indicates that there is no obligate link between Mb and oxidative phosphorylation. The scaling of muscle and cardiovascular properties indicates that Mb is expected to show a smaller role in O_2 supply in mice than in the muscles of larger mammals. More interesting, however, is the possibility that changes in the underlying muscle fibre properties during development without myoglobin may eliminate an O_2 supply deficit and minimize the need for Mb-mediated O_2 supply. Thus, a simple analysis of the intracellular diffusion problem not only helps to understand the physical basis of Mb-mediated O_2 supply but also to predict the changes in muscle properties necessary for full aerobic function in the absence of Mb.

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