The Journal of Experimental Biology 209, 2385-2394
Published by The Company of Biologists 2006
doi:10.1242/jeb.02287

Review

Partitioning locomotor energy use among and within muscles

Muscle blood flow as a measure of muscle oxygen consumption

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Accepted 19 April 2006

Summary

Linking the mechanics and energetics of locomotion in vertebrates has been hampered by a lack of information regarding the energy use of individual skeletal muscles *in vivo*. Here, we present a review of the available data concerning the relationship between the rates of skeletal muscle blood flow and oxygen consumption ($\dot{V}_{O_2}$). In active muscle, during aerobically supported exercise, there is a linear relationship between these variables, irrespective of the muscle fiber type and intensity of exercise through most of the aerobic exercise range. We conclude that the rate of blood flow is the best available indicator of aerobic metabolic rate in multiple individual muscles or regions of muscles during locomotion. The practical considerations of using the injectable microsphere technique to measure muscle blood flow in this context are discussed.

Key words: blood flow, muscle energy use, exercise, hyperemia, fiber type, locomotion.

Introduction

Linking the mechanics and energetics of locomotion in vertebrates has been hampered by lack of information regarding the energy use of individual skeletal muscles *in vivo*. Organismal energy use by animals and humans during locomotion has received considerable attention, and a reasonable inference is that the majority of the increase in metabolism during locomotion results from increases in the metabolism of active locomotor muscles with smaller contributions from increases in metabolism of the heart and respiratory muscles. However, when the goal is to connect the mechanical function of the muscles to energy use, knowing total energy use by the active locomotor muscles provides limited guidance. In some cases, e.g. flight metabolism of birds and bats, most of the metabolic energy use may be reliably ascribed to a limited number of muscles. However, even in these cases the distribution of energy use among and within these large muscles and the amount of energy used by accessory muscles remain unknown. The problem is particularly acute when attempting to understand the mechanical determinates of energy use during legged locomotion, which involves numerous muscles with diverse mechanical functions. Our understanding of these systems would be significantly enhanced by the ability to partition overall energy use among and within the active muscles.

Despite the obvious utility of measuring the energy use of the individual skeletal muscles used during locomotion, technical difficulties have hampered these measurements. Direct measurements of oxygen consumption ($\dot{V}_{O_2}$) of individual muscles during locomotion using the Fick method are likely not feasible with current technology except under very limited circumstances. These measurements require measures of arterial oxygen content, the oxygen content of venous blood emerging from the individual muscle, and the rate of blood flow to the same muscle. Many hurdles stand in the way of these measurements, including the presence of numerous collateral branches in the circulation, which makes measuring the average venous oxygen content of blood from an individual muscle difficult.

Indirect methods that indicate recruitment of active muscle fibers cannot be correlated with any certainty to metabolic energy use. Electromyographic (EMG) activity is the most commonly used measure of muscle activity. EMG activity is valuable for indicating timing and relative recruitment within a given region of a muscle, but the results are difficult to relate quantitatively to energy use. Quantitative interpretation of EMG activity probably works best for individual muscle

regions operating under similar mechanical conditions. Technical difficulties in quantification stem from multiple sources including variation in the size of the electrodes and their placement, which together determine the active muscle volume sampled. The relation between electrical activity and energy use is also influenced by the mechanical behavior of the muscle, because energy use varies with shortening speed and duty cycle (Kushmerick, 1983; Ferguson et al., 2001; Hamann et al., 2005). Added to these difficulties, is the simple fact that simultaneous EMG recording from a large number of muscles may require extensive surgery to ensure accurate electrode placement. Glycogen depletion measured biochemically, or visualized histochemically, has also been used to indicate muscle fiber recruitment. However, glycogen use does not increase uniformly with increasing energy demand in muscle, but instead is highest under conditions approaching or exceeding the aerobic capacity of the active muscle fibers (Holloszy et al., 1998; Brooks, 1998). Thus, this technique can indicate the relative volume of active muscle fibers, particularly under conditions of high-energy demand (Armstrong and Laughlin, 1985; Armstrong et al., 1986) or prolonged fatiguing contractions (English and Weeks, 1987), but cannot with any certainty be quantitatively related to aerobic energy use. Similarly, using magnetic resonance imaging to measure the transverse relaxation time estimates the volume of muscle that was active during a preceding exercise bout (Meyer and Prior, 2000; Kinugasa et al., 2005), but cannot specify the amount of energy used within this volume.

Muscle blood flow and oxygen consumption in vertebrate skeletal muscle

Marsh et al. suggested that the technique with the best potential for accurately assessing energy use in vertebrate muscles during locomotion is the measurement of blood flow to the muscles (Marsh et al., 2004). Measurements of blood flow to all the individual active muscles, or portions of these muscles, can be made simultaneously using the well-established microsphere technique (Buckberg et al., 1971; Bassingthwaighte et al., 1987; Bassingthwaighte et al., 1990; Kowallik et al., 1991; Glenny et al., 1993). Conceptually, this technique is quite simple, although it can be technically challenging to implement. Microspheres, labeled radioactively or with a dye, are injected into the systemic circulation, usually *via* the left ventricle or left atrium. The microspheres mix with the blood and provide a tracer for the distribution of flow. The size of microspheres, typically 15 μm, is chosen so that they will lodge in the systemic capillaries. Thus, the number of microspheres that lodge in a particular volume of tissue is proportional to the blood flow to that tissue volume. The number of microspheres in the tissue can be converted to flow rate because during the injection a reference arterial blood withdrawal is done at a known constant rate. This reference withdrawal can also be used to calculate the total cardiac output by simple dilution principles, given an accurate estimate of the number of spheres injected.

Over 20 years ago, Armstrong and Laughlin examined the use of this technique as a potential measure of muscle fiber recruitment (Armstrong and Laughlin, 1985), but were cautious about its use because they considered that factors other than local metabolic rate might play a significant role in determining blood flow. However, our conclusion from a broad array of literature is that within acceptable limits, the amount of blood flow to an active skeletal muscle is proportional to its $\dot{V}_{O_2}$, and therefore this technique represents the best currently available method for estimating aerobic energy use by individual muscles in freely moving animals. Our intention here is to provide a brief overview of this literature considering various physiological and technical issues that could potentially compromise the accuracy of the technique. The literature on cardiac output and muscle blood flow is extensive and our aim is not to be comprehensive, but to cover the relevant issues with representative citations.

Control of blood flow to active skeletal muscle

The proportionality between skeletal muscle $\dot{V}_{O_2}$ and blood flow results from control systems that link local metabolic rate in skeletal muscle to the rate of flow through the local microcirculation. The available evidence suggests that blood flow to active skeletal muscles is controlled so that the rate of oxygen delivery is proportional to the metabolic rate (Delp and Laughlin, 1998; Murrant and Sarelius, 2000; Laughlin and Korzick, 2001; Boushel, 2003; Ellsworth, 2004; Segal, 2005). Central mechanisms play a part in this control, perhaps restraining flow during periods of high demand, but local mechanisms that control flow through relatively small microvascular units appear to play the dominant role under many conditions (Segal, 2005). However, even given the dominance of local control, the possibility exists that the blood flow (or oxygen delivery) may not vary directly with the change in oxygen consumption. For example, some studies have suggested that at low levels of aerobic effort alterations in the microvasculature might lead to changes in oxygen extraction with little or no change in blood flow, and only with further increases in metabolic rate is elevated flow necessary to supply oxygen demand (for a review, see Segal, 2005). Investigators have also suggested that, when demand for flow is great, increases in flow may be constrained by central sympathetic output, which could alter the relation between muscle blood flow and $\dot{V}_{O_2}$ (Mortensen et al., 2005).

In interpreting the correlation between muscle blood flow and $\dot{V}_{O_2}$, one should bear in mind that the regulated variable in the microcirculation of skeletal muscle is oxygen delivery, not blood flow *per se* (Rowell et al., 1986; Ferretti et al., 1992; Koskolou et al., 1997; Gonzalez-Alonso et al., 2006). Thus, the relation between muscle blood flow and $\dot{V}_{O_2}$ is dependent on the carrying capacity of the arterial blood, the saturation of hemoglobin with oxygen, and the oxygen extraction efficiency. When total arterial oxygen content remains constant, oxygen delivery will be proportional to flow, but experimental (Gonzalez-Alonso et al., 2006) or natural (Longhurst et al., 1986; Taylor et al., 1987; Weber et al., 1987) mechanisms that

alter arterial oxygen content, will alter the amount of flow needed to achieve an appropriate oxygen delivery. Changes in arterial oxygen content do not prevent the use of blood flow as an indicator of the $\dot{V}_{O_2}$ of active muscle, they simply make the conversion of flow to energy use more complex because the change in flow must first be converted to a change in oxygen delivery.

Response of cardiac output to increasing organismal $\dot{V}_{O_2}$ during exercise

Initial support for the hypothesis that muscle blood flow is linearly related to muscle $\dot{V}_{O_2}$ comes from numerous studies of organismal $\dot{V}_{O_2}$ and cardiac output in diverse animals over a broad range of exercise intensities below $\dot{V}_{O_2max}$. Cardiac output increases linearly with organismal $\dot{V}_{O_2}$ in birds (Grubb, 1982; Grubb et al., 1983; Ellerby et al., 2005), mammals, including humans (Barger et al., 1956; Stenberg et al., 1967; Musch et al., 1985; Snyder et al., 1999), and fish (Webber et al., 1998). This relation is largely independent of the volume of muscle recruited or the form of exercise conducted (Stenberg et al., 1967; Bezucha et al., 1982). The slope of the relation depends on the oxygen carrying capacity of the blood and is also different in different vertebrate groups (Grubb, 1982; Webber et al., 1998; Synder et al., 1999; Ellerby et al., 2005). The simplest explanation of a linear relation of cardiac output and organismal $\dot{V}_{O_2}$ is that the increase in blood flow supplies the increased $\dot{V}_{O_2}$ of the heart and active skeletal muscle fibers, while other tissues, which have lower extraction efficiencies, continue to receive approximately the same flow as at rest (Wolff, 2003). This explanation is known not to be strictly true in all cases because some species of mammals and birds exhibit decreased blood flow to the abdominal organs, thus redistributing flow to working muscles (Rowell, 1974; Armstrong and Laughlin, 1984; Manohar, 1986; Armstrong et al., 1987a; Butler et al., 1988). The largest contribution of this redistributed flow in supplying muscle blood flow during exercise has been estimated in untrained humans, in whom the redirected flow could supply approximately 15% of the muscle flow (Rowell, 1974). In trained humans the contribution declines to 10% (Rowell, 1974) and in other mammals the contribution is lower, e.g. approximately 5% in miniature swine (Armstrong et al., 1987a). In some species of mammals and birds, including dogs and guinea fowl, little or no redistribution of flow from splanchnic and renal circulations has been found (Rowell, 1974; Fixler et al., 1976; Ellerby et al., 2005). Some flow is also redistributed from non-exercising muscle (Rowell, 1974; Butler et al., 1988; Ellerby et al., 2005). Despite these complications, the contribution of the redistributed blood flow to the increase in flow to the exercising muscles is modest. Therefore, the linear increase in cardiac output with increasing organismal $\dot{V}_{O_2}$ in a diverse array of vertebrates supports the idea that the blood flow and $\dot{V}_{O_2}$ in active skeletal muscle are closely related because the increase in cardiac output during exercise to a large extent reflects the sum of the increases in flow to active skeletal muscles.

Blood flow and $\dot{V}_{O_2}$ in groups of active muscles

A more direct assessment of the reliability of using muscle blood flow as an indicator of muscle $\dot{V}_{O_2}$ can be made by examining these variables in groups of active muscles. Two types of studies bear on this question: (1) animal exercise studies measuring organismal $\dot{V}_{O_2}$ and muscle blood flow, and (2) studies of the exercising arms or legs of humans, measuring regional $\dot{V}_{O_2}$ with the Fick method and blood flow using thermal or dye dilution techniques.

Blood flow to the active skeletal muscles measured using microspheres can be compared reliably to the increase in organismal $\dot{V}_{O_2}$ if two criteria are met: (1) the increase in $\dot{V}_{O_2}$ consists almost entirely of the increase in $\dot{V}_{O_2}$ of the active muscles, and (2) total blood flow to all of the active skeletal muscles is measured.

The first criterion is difficult to assess completely, but with the exception of increases in energy use by the heart, the oxygen consumption by tissues other than active skeletal muscles is unlikely to increase going from rest to exercise, and likely remains approximately constant. The decrease in blood flow to the abdominal organs that occurs in some species might suggest substantial decreases in energy use by these organs, but oxygen extraction by these organs is low at rest and increases substantially during exercise, allowing oxygen uptake to remain approximately constant (Rowell et al., 1964; Rowell, 1974; Takala, 1996).

Few studies meet the second criterion. Although microspheres have been used to assess blood flow to exercising muscles in numerous studies, few investigations have measured the total flow to all the active muscles; more typically, mass-specific flow to a subset of muscles has been reported. The most complete studies are those measuring blood flow to the leg muscles in guinea fowl during walking and running (Marsh et al., 2004; Ellerby et al., 2005; Ellerby and Marsh, 2006; Rubenson et al., 2006). The results of these studies show an excellent correlation of mean total blood flow to the leg muscles, and mean organismal $\dot{V}_{O_2}$ (Fig. 1). Data on miniature swine (Armstrong et al., 1987a) also show a good correlation of total muscle blood flow and organismal $\dot{V}_{O_2}$, but in these data the slope appears to change at high levels of $\dot{V}_{O_2}$. Although this study on miniature swine is the most complete study other than those using guinea fowl, sampling biases could have influenced the assessment of total muscle flow. The major goal of Armstrong et al.'s study (Armstrong et al., 1987a) was to assess variability in mass-specific blood flow among and within skeletal muscles varying in fiber-type composition. They sampled two muscles systematically, and many other muscles with small samples relative to the muscles' total mass. Total muscle flow was calculated by averaging the mass-specific flows from these samples and multiplying by an estimate of total muscle mass. Because of the large variation in mass-specific flow within and among muscles, averaging the mass-specific data without weighting them for the muscle mass could lead to biased estimates of total flow.

Studies of blood flow to the working muscles in the arms or legs of humans offer the advantage of simultaneous measures

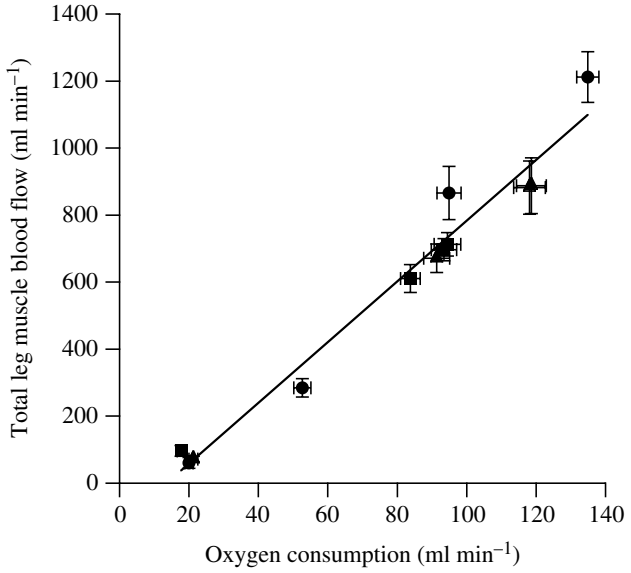

Fig. 1. Relation between mean blood flow to the leg muscles and mean organismal oxygen consumption in guinea fowl. The line is a regression line through all the data (slope=9.1±1.2, 95%C.I.). Values are means ± 1 s.e.m. (*N*=5-8). Circles (Marsh et al., 2004); triangles Rubenson et al., 2006); squares (Ellerby and Marsh, 2006).

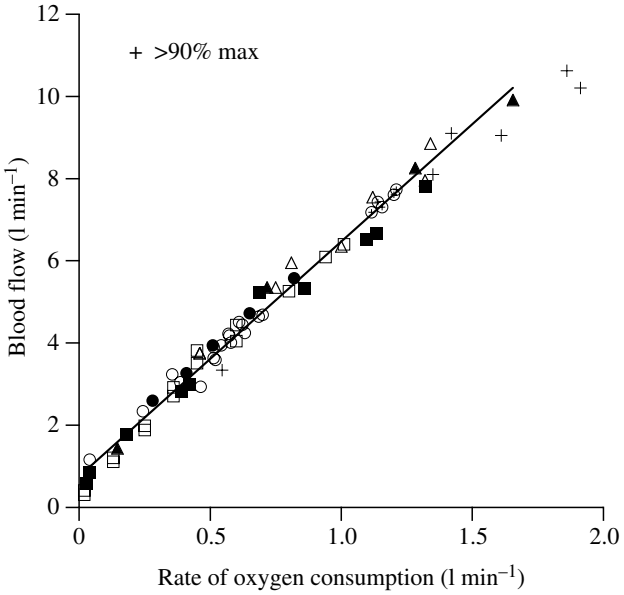

Fig. 2. Relation between regional blood flow and oxygen consumption in the legs or arms of humans. Symbols indicate data taken from different references as indicated in the legend. The line is a regression line (slope=5.72±0.22, 95%C.I.) through all the data except those collected at greater than 90% of $\dot{V}_{O_2max}$ in short incremental exercise tests (see text for details). Filled circles (Anderson and Saltin, 1985); open circles (Ferguson et al., 2001); filled squares (Calbet et al., 2005); open squares (Voliantis et al., 2004); filled triangles (Mortensen et al., 2005); open triangles (Richardson et al., 1995); crosses in circles (Golzalez-Alonso et al., 1998).

of blood flow and $\dot{V}_{O_2}$ to the same region of the body, rather than relying on the change in organismal $\dot{V}_{O_2}$ to estimate the metabolism of the muscles. The data from these studies for levels of exercise requiring a $\dot{V}_{O_2}$ of less than 90% of the $\dot{V}_{O_2max}$ of the muscle group under study demonstrate a linear relation between blood flow and $\dot{V}_{O_2}$ with an impressive degree of agreement across a range of studies (Fig. 2). Despite the differences among some groups noted in these studies, the overall agreement of the data is striking given that they were obtained from studies of: different muscle groups (whole arm, whole leg, or knee extensors only); steady state and incremental exercise protocols; different forms of exercise (skiing, cycling, isolated knee extension, or arm cranking); and from various subjects with different degrees of training. Data obtained when the subjects were near $\dot{V}_{O_2max}$ in incremental protocols (Calbet et al., 2005; Mortensen et al., 2005) tend to show lower blood flow for a given $\dot{V}_{O_2}$, but even these data do not fall very far off the regression line through the remaining data (Fig. 2). These regional blood flow studies in humans support strongly our suggestion that the increase in blood flow delivered to an active muscle is closely matched to its increase in $\dot{V}_{O_2}$ up to at least 90% of the $\dot{V}_{O_2max}$ of the muscles.

Muscle blood flow and fiber type

Even though the overall blood flow to the active skeletal muscles in an animal is proportional to $\dot{V}_{O_2}$, one might question whether this relation holds for muscles, or regions of muscles, that vary in fiber type composition. *A priori*, muscles with larger proportions of oxidative fibers that have greater capillary densities might be predicted to have greater extraction efficiencies. One piece of evidence against this prediction is the linear relation of total blood flow and $\dot{V}_{O_2}$ found in guinea fowl and humans (Figs 1 and 2). Past work has shown that high-oxidative slow fibers (Type 1) are recruited at low exercise intensities, followed by high-oxidative fast fibers, and then by low oxidative fast fibers (Laughlin and Armstrong, 1982; Armstrong et al., 1987a). This recruitment order is very likely followed in guinea fowl as well (Ellerby et al., 2005), and apparently, the relation of flow to $\dot{V}_{O_2}$ does not change markedly as faster and less oxidative fibers are recruited (Figs 1 and 2). Additionally, Ferguson et al. examined the effects of contraction frequency (Ferguson et al., 2001), which might be expected to alter the fraction of fast fibers recruited, and their data show the same ratio of flow to $\dot{V}_{O_2}$ at both contraction frequencies. More direct evidence for a similar relation of muscle blood flow and $\dot{V}_{O_2}$ in different fiber types comes from recent *in situ* studies in rats using an innovative technique for estimating $\dot{V}_{O_2}$ (Behnke et al., 2003; McDonough et al., 2005). The soleus, which is composed of primarily slow fibers, has a higher resting blood flow compared to its $\dot{V}_{O_2}$ than do muscles composed of mostly fast fibers. However, the relation between flow and $\dot{V}_{O_2}$ in active fibers is similar in the two fiber types (Fig. 3).

The constancy of oxygen extraction across varying fiber types is consistent with the conclusion that diffusion of oxygen

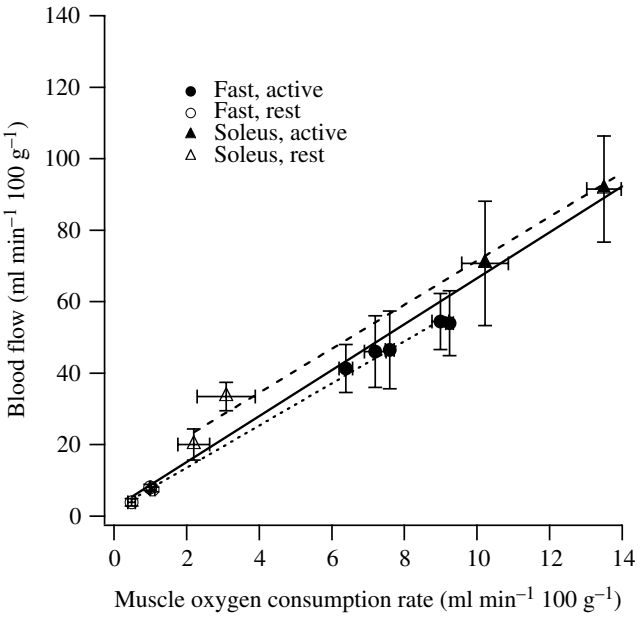

Fig. 3. Relation between blood flow and oxygen consumption of individual muscles in rats during *in situ* measurements. Data are from (Behnke et al., 2003; McDonough et al., 2005). The solid line is a regression through all the data (slope=6.4±0.7, 95%C.I.); broken line, through the data for the soleus only (slope=6.1±1.1, 95%C.I.); stippled line, through the data for fast muscles only (slope=5.9±0.4, 95%C.I.). Values are means ± 1 s.e.m. (*N*=6 or 20).

under normal circumstances is limited by the characteristics of the capillaries, and is independent of fiber size (Wagner, 2000), and with the relatively constant relations between capillarity, mitochondrial volume, and $\dot{V}_{O_2}$ (Hoppeler and Weibel, 2000). Thus, although abundant evidence indicates that maximal mass-specific blood flow is higher to fibers with higher oxidative capacities, this flow appears to be proportional to the higher metabolic rates of these fibers. The available data, therefore, suggest that increases in blood flow to active skeletal muscle can be used to indicate aerobic energy expenditure independent of fiber type.

Muscle blood flow and duty cycle

The mode of muscle use during exercise might also be predicted to alter the relation of blood flow to $\dot{V}_{O_2}$. Muscle blood flow is temporarily reduced during contraction, and the relation of flow to $\dot{V}_{O_2}$ might be altered by differences in venous pumping due to differing types of contractions. However, the available evidence does not bear out this prediction. The relation of cardiac output to $\dot{V}_{O_2}$ has been found to be approximately the same in static and dynamic exercise in humans (Bezucha et al., 1982). Either static or dynamic repetitive contractions can change the time course of the response to increasing exercise intensity (Laughlin and Joyner, 2003; Sheriff, 2003), alter the relative contribution of heart rate and stroke volume to cardiac output (Bezucha et al., 1982), and cause marked pulsations in the flow during the contraction–relaxation cycle (Eriksen et al., 1990; Rådegran, 1997). However, the time-averaged flow for a given $\dot{V}_{O_2}$ appears largely unaltered by the frequency or duty cycle (Bezucha et al., 1982; Lewis et al., 1983; Ferguson et al., 2001).

Variation in blood flow with exercise duration

How quickly is the relation between flow and $\dot{V}_{O_2}$ established at the onset of exercise, and does the relation between these variables remain constant with exercise duration?

In some animal studies, muscle blood flow has been reported to increase at the beginning of exercise, independent of metabolic rate; however, any dissociation of flow and $\dot{V}_{O_2}$ is very brief (Laughlin and Armstrong, 1982). In humans, in whom the kinetics might be expected to be slower than smaller animals, cardiac output and $\dot{V}_{O_2}$ are correlated with only a short (10–45 s) lag during exercise transitions (De Cort et al., 1991; Bangsbo, 2000), and time constants for the change in leg blood flow and metabolic rate are not significantly different (Paterson et al., 2005).

Muscle blood flow has been reported to increase with prolonged exercise in rats (Laughlin and Armstrong, 1983); however, this study was done without accompanying measurements of metabolic rate, and the $\dot{V}_{O_2}$ may have increased with exercise duration. Shifting patterns of flow during prolonged exercise might be expected as fibers fatigue and other fibers are recruited. Cardiac output and muscle blood flow were reported to increase proportionately more than organismal $\dot{V}_{O_2}$ during prolonged exercise in miniature swine (Armstrong et al., 1987b). However, muscle blood flow measured by Armstrong et al. was actually quite constant in most muscles until 30 min of exercise, which was close to the time the animals fatigued (Armstrong et al., 1987b). A subsequent study with this same species showed that muscle blood flow remains constant as long as body temperature is maintained (McKirnan et al., 1989). In humans under certain exercise conditions, blood flow increases with exercise duration, but this increased flow correlates well with an increase in muscle $\dot{V}_{O_2}$ (González-Alonso, 1998; Ferguson et al., 2001).

Blood flow and $\dot{V}_{O_2}$ in resting muscle

Extraction of oxygen by resting muscle is lower than active muscle, both pre- or post-exercise (Bangsbo and Hellsten, 1998; Clark et al., 2000), and resting flow and resting metabolic rate may not be well correlated (Behnke et al., 2003; McDonough et al., 2005). The distribution of this resting flow and what happens to this flow during exercise will determine whether the resting flow should be subtracted from the exercise flow in estimating the metabolic rate attributable to an individual active muscle or muscle group. If all the flow to the resting muscle goes through vessels exchanging oxygen and nutrients with the muscle fibers, then oxygen extraction in this microcirculation obviously is quite different at rest than during activity. In which case, an argument could be made for not subtracting the resting flow. However, Clark et al. review

evidence that a portion of the resting flow goes through vessels that are not effectively exchanging oxygen or nutrients with the muscle fibers (Clark et al., 2000). In this case, whether the resting flow should be subtracted from the active flow, depends on what proportion of the resting flow is non-nutritive and whether this flow remains constant when the muscle becomes active. The available data have insufficient resolution to answer these questions. For example, the *in situ* data on fast and slow muscles of the rat (Behnke et al., 2003; McDonough et al., 2005) indicate that the mass-specific resting blood flow to the largely slow-fibered soleus is much higher than that to the fast muscles studied (Fig. 3). Despite the higher resting flow, the slope of the increase in flow is quite similar when the data for the soleus and that for the fast muscles are considered separately (Fig. 3), and this would argue for using the net increase in flow when correlating blood flow and energy use (Ferreira et al., 2005). However, given the variability of the data, a regression through all of the data also describes the overall relation quite well (Fig. 3).

Complicating this issue when studying flow *in vivo*, is the question of whether during the 'resting' flow measurements a particular muscle of interest is actually inactive. For example, in treadmill studies of mammals in which resting measurements are taken with the animal standing (Laughlin and Armstrong, 1983; Armstrong et al., 1987a), a subset of muscle fibers is active to maintain the standing posture and this activity is expected to be differentially distributed among different muscles according to fiber type. This effect can be clearly seen in Delp and Armstrong's data (Delp and Armstrong, 1988), who found substantially lower blood flows during standing in the soleus and oxidative portion of the gastrocnemius after denervation of these muscles in rats. In guinea fowl, we have attempted to minimize the activation of postural muscles by performing resting measurements with the bird 'sitting' in a darkened box (Ellerby et al., 2005), but we do not know the extent to which the birds rely on postural muscles to maintain this position.

Fortunately, even for moderate increases in exercise intensity, the active flow to many skeletal muscles is much higher than the resting flow, and similar conclusions are reached about the distribution of exercise metabolism among the muscles whether total or net flows above rest are considered. For example, at the lowest treadmill speed at which guinea fowl were tested (0.5 m s^{-1}), the swing phase muscles receive 25% of the total flow (Marsh et al., 2004), and 24% of the net increase in flow above rest.

Blood flow as a function of $\dot{V}_{O_2}$ at low levels of exercise

A conclusion sometimes cited from some previous work on muscle blood flow is that small increases in $\dot{V}_{O_2}$ in muscle can be accommodated by changing oxygen extraction with little or no increase in flow (e.g. Segal, 2005). This idea, if correct, might call into question using blood flow as an *in vivo* indicator of the increase in muscle $\dot{V}_{O_2}$ during low-intensity exercise. The evidence for this conclusion appears to stem mostly from some *in situ* studies of muscle blood flow and $\dot{V}_{O_2}$ (Stainsby and Otis, 1964; Belloni et al., 1979), and from changes in oxygen extraction in human muscle with increasing exercise intensity (Andersen and Saltin, 1985). However, data from neither type of study provides convincing evidence that increasing $\dot{V}_{O_2}$ in muscle is independent of increasing blood flow at low levels of $\dot{V}_{O_2}$. Stainsby and Otis, using an *in situ* preparation (Stainsby and Otis, 1964), examined the effect of reduced arterial oxygen content on flow during muscle stimulation. Their data show a relatively stable $\dot{V}_{O_2}$ until a critical value of arterial oxygen content is reached. However, before that value is reached blood flow increases with decreasing arterial oxygen content and the level of oxygen delivery, calculated from the measured flow and oxygen content, remains within 10% of the initial value. No change in flow with increasing $\dot{V}_{O_2}$ was found in an *in situ* dog muscle preparation over a range of twitch frequencies from 0.25 to 1 Hz (Belloni et al., 1979). However, the resting flows measured by Belloni et al. were rather high, and other studies show simultaneous increases in flow and $\dot{V}_{O_2}$ using similar preparations (Horstman et al., 1976; Young and Sparks, 1980). Also, stimulation rates below 1 Hz are well below any realistic *in vivo* stimulation frequency. Studies of human muscle show no evidence of increases in $\dot{V}_{O_2}$ without corresponding increases in blood flow (Fig. 2). In fact, a regression through the values of blood flow and $\dot{V}_{O_2}$ extrapolates slightly above the measured values of blood flow at rest (Fig. 2), suggesting that initial increases in flow at very low values of $\dot{V}_{O_2}$ may be actually slightly larger than would be expected. Finally, studies of blood flow during legged locomotion in rats, miniature swine, dogs and guinea fowl all show increases in blood flow to the leg muscles at the lowest walking speeds measured (Laughlin and Armstrong, 1982; Armstrong et al., 1987a; Musch et al., 1987; Marsh et al., 2004; Ellerby et al., 2005).

Metabolic rate and blood flow in tissues other than skeletal muscle

Blood flow and metabolic rate are correlated in some non-muscular organs, but not in others. Blood flow to the brain is closely correlated with metabolic rate (for a review, see Girouard and Iadecola, 2006). In contrast, the relation of blood flow to oxygen uptake in the splanchnic and renal circulations changes dramatically between rest and exercise. At rest, splanchnic flow varies with metabolic demand and oxygen extraction efficiency remains low, but during exercise blood flow may be reduced substantially while $\dot{V}_{O_2}$ remains relatively constant because of increases in extraction efficiency (Rowell, 1974). Regulation of blood flow to these abdominal organs reflects functional demands for flow that are substantially different than those for skeletal muscle (Gallavan and Chou, 1985; Regan et al., 1995). In the mammalian heart, changes in the extraction of oxygen are limited, but do play a role in supplying oxygen to the cardiac fibers at high levels of demand in humans (Feigl, 1983) and in dogs, especially in the right ventricle (Hart et al., 2001). If similar changes in extraction also occur in the avian heart, they might explain the flattening of the increase in blood flow

to the ventricles of guinea fowl as exercise intensities approach $\dot{V}_{O_2max}$ (Ellerby et al., 2005).

Accuracy of the microsphere measures of blood flow

The microsphere technique allows blood flow to be measured simultaneously to all tissues in the body in which the spheres are trapped during the first pass through the systemic circuit. The size of the microspheres is important in this context, and spheres with a diameter of 15 μm are trapped in the capillaries of most tissues in mammals, birds and fish (Fan et al., 1979; Wolfenson, 1983; Schultz et al., 1999; Ellerby et al., 2005). Microsphere measures of blood flow have been extensively validated; however, implementation in a new system requires validation to determine the doses of spheres to be injected and to assure that accurate recovery from the tissues is attained [see methods described elsewhere (Ellerby et al., 2005)]. Briefly, the accuracy of the technique depends on the following:

(1) Accurate measurements of the numbers of microspheres trapped in a tissue sample. Previous work has indicated that at least 400 spheres must be recovered in a tissue sample to allow for errors in measured flow to less than 10% (Buckberg et al., 1971), although smaller numbers of spheres may be acceptable if larger numbers of spheres are present in the reference withdrawal (Nose et al., 1985). Having larger numbers of spheres in the tissue samples does not necessarily reduce the errors below 10% (Buckberg et al., 1971). Non-random distribution of microspheres in the microcirculation is apparently not an issue until tissue samples become quite small, probably less than 50 mg (Bassingthwaighte et al., 1987), although at microscopic scales distribution is non-random (Decking et al., 2004). When dye-labeled spheres are used, the tissue counts should also be corrected for losses in the extraction process (Chien et al., 1995).

(2) An accurate and uniform reference blood withdrawal rate. The withdrawal rate of arterial blood is important to the accuracy of the blood flow measurements, because all the flow rates and the cardiac output are calculated using the number of spheres in this reference sample. The withdrawal rate must remain constant during the entire time that spheres are being injected. In a freely moving animal, maintaining a uniform withdrawal can be problematic because movement of the cannula can cause it to be transiently occluded by the arterial wall. Because of the importance of an accurate reference withdrawal, the manual of the Fluorescent Microsphere Resource Center at University of Washington (http://fmrc.pulmcc.washington.edu/fmrc.shtml) recommends that two simultaneous reference blood samples be drawn if possible. However, this procedure is often not implemented because it is not surgically practical, and results in larger volumes of blood being withdrawn. Because of the potential for systematic variation in the measured flow across animals, implementing statistical analyses that partition the sources of variability are useful in detecting the effects of the experimental treatments (Ellerby et al., 2005). Fortunately, errors in the withdrawal rate of the reference sample cause the same proportional error in calculating flow to all the muscles, and do not affect calculations of the fractional distribution of the total flow. Consequently, the variability among different animals in the fraction of the total flow distributed to the individual muscles may be smaller than the variability in absolute flow (Marsh et al., 2004; Ellerby et al., 2005). The fraction of the total flow distributed to a muscle, or a group of muscles, determines the estimate of the fraction of the locomotor cost attributable to a muscle or group of muscles (Marsh et al., 2004; Ellerby et al., 2005; Ellerby and Marsh, 2006; Rubenson et al., 2006).

(3) The injected spheres are well mixed with the blood leaving the heart. Previous work in mammals indicates that mixing is somewhat better when the spheres are injected into the left atrium, but that mixing is adequate when injections are done in the left ventricle (Buckberg et al., 1971). With adequate mixing, one expects that on average the flow to the tissues on the right and left sides will be equal. In mammalian studies, the kidneys are often used to examine whether flow is equally distributed to the right and left sides. However, in birds, and probably other vertebrate groups, using this organ to assess bilateral flow may be problematical. The renal circulation in these animals is complex due to the presence of renal portal flow. The amount of renal portal flow can vary due to active valving (Odlind, 1978), and renal portal flow in birds can have variable numbers of microspheres representing those that have passed through arterio-venous shunts in the legs (Wolfenson, 1983). Experimental conditions may sometimes necessitate injection into a systemic artery. For example, the circulatory plan of fish requires that the microspheres be injected into the systemic arterial system following the circulation to the gills (Kolok et al., 1993; Wilson and Egginton, 1994; Taylor et al., 1996; Schultz et al., 1999).

(4) The circulation is not compromised by the injection of too many spheres. Blockage of the capillaries in skeletal muscle can be calculated based on capillary numbers, and successive injections under the same exercise conditions can validate that the muscle flow measurements do not depend on injection order (Ellerby et al., 2005). Of course, other critical parts of the circulation may become compromised, e.g. the microcirculation in the CNS, and the overall exercise performance of the animal should be compared to its normal performance.

We conclude that the various sources of error in measuring flow with microspheres make the technique suitable for measuring the mean flow to a muscle or group of muscles under a given exercise condition, but make it less useful for measuring variation in flow among individual animals. Certainly, in guinea fowl mean total flow to the legs varies among exercise conditions as expected from changes in $\dot{V}_{O_2}$ (Fig. 1), despite substantial variation in individual values [see fig. 1 in Ellerby et al. (Ellerby et al., 2005)].

Conclusion

During aerobic exercise, the rate of oxygen delivery to active muscle tissue is matched to the tissue's $\dot{V}_{O_2}$ primarily by local mechanisms. Therefore, as long as arterial oxygen content remains constant, the rate of blood flow to muscle correlates well with muscle $\dot{V}_{O_2}$. Irrespective of differences in exercise intensity, muscle fiber type and mechanical function, the available data show that there is an approximately linear relationship between muscle $\dot{V}_{O_2}$ and blood flow rate. Consequently, changes in muscle blood flow can be used quantitatively to indicate which muscles are responsible for increasing aerobic energy expenditure during exercise. If arterial oxygen content is altered by exercise, e.g. due to splenic contraction, increases in muscle blood flow presumably can still be used to indicate increasing muscle $\dot{V}_{O_2}$ as long as the change in oxygen delivery is taken into account. The injectable microsphere technique simultaneously measures blood flow to all parts of the body and allows the partitioning of organismal energy use among individual skeletal muscles, or regions of muscles, to be determined. These measurements remove a major constraint on linking the mechanics and energetics of locomotion during aerobic exercise, because the energetic costs of specific mechanical functions need no longer be inferred from whole organism energy expenditure, but can be based on measurements at the muscle level.

This study was supported by NIH grant AR47337 to R.L.M.

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