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EXPERIMENTAL PHYSIOLOGY: HOT TOPIC

SKELETAL MUSCLE CAPILLARY FUNCTION: CONTEMPORARY OBSERVATIONS AND NOVEL HYPOTHESES

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Abstract

The capillary bed constitutes a vast surface facilitating exchange of O_2, substrates and metabolites between blood and organs. In contracting skeletal muscle capillary blood flow and O_2 diffusing capacity as well as O_2 flux may increase two orders of magnitude above resting. Chronic diseases such as heart failure, diabetes and also sepsis impair these processes leading to compromised energetic, metabolic and ultimately contractile function. Among researchers seeking to understand blood-myocyte exchange in health and the bases for dysfunction in disease there is a fundamental disconnect between microcirculation specialists and many physiologists and physiologist clinicians. Whereas the former observe capillaries and capillary function directly (muscle intravital microscopy) the latter generally use indirect methodologies (e.g., post-mortem tissue analysis, 1- methyl xanthine, contrast enhanced ultrasound, permeability surface area product) and interpret their findings based upon August Krogh's observations made nearly a century ago. "Kroghian" theory holds that only a small fraction of capillaries support red blood cell (RBC) flux in resting muscle leaving the vast majority to be "recruited" (i.e., initiate RBC flux) during contractions which would constitute the basis for increasing capillary exchange surface area and reducing capillary-mitochondrial diffusion distances. Experimental techniques each have their strengths and weaknesses and often the correct or complete answer to a problem emerges from integration across multiple technologies. Today Krogh's entrenched "capillary recruitment" hypothesis is challenged by direct observations of capillaries in contracting muscle; something that he and his colleagues could not do. Moreover, in the peer-reviewed scientific literature, application of a range of contemporary physiological technologies including intravital microscopy of contracting muscle, magnetic resonance and near infrared spectroscopy and phosphorescence quenching combined with elegant *in situ* and *in vivo* models suggest that the role of the capillary bed, at least in contracting muscle, is subserved without the necessity for *de novo* capillary recruitment of previously non-flowing capillaries. When viewed within the context of the capillary recruitment hypothesis, this evidence casts serious doubt on the interpretation of those data that are based upon Kroghian theory and indirect methodologies. Thus today a wealth of evidence calls for a **radical revision of blood-muscle exchange theory** to one in which most capillaries support RBC flux at rest and, during contractions, capillary surface area is "recruited" along the length of previously flowing capillaries. This occurs, in part, by elevating capillary hematocrit and extending the length of the capillary available for blood-myocyte exchange (i.e., longitudinal recruitment). Our understanding of blood-myocyte O_2 and substrate/metabolite exchange in health and the mechanistic bases for dysfunction in disease demands no less.

Introduction

The capillary bed presents a prodigious surface area facilitating blood-tissue interchange of O_2, substrates and metabolites as well as hormones and other bioactive/signaling molecules. Of all capillary beds that of skeletal muscle represents by far the largest and, especially during exercise, plays the dominant role in whole body O_2, glucose, lactate and fatty acid dynamics. Access to the capillary surface is not the same for red blood cells (RBCs) versus plasma because, in the microcirculation, RBCs and plasma interact differently with the endothelial surface layer (termed "glycocalyx", by some) and travel at different speeds resulting in a far lower capillary (e.g., 15%) than systemic (i.e., 45%) hematocrit (**Klitzman & Duling, 1979; Sarelius & Duling, 1982; Poole *et al*. 1997; Frisbee & Barclay, 1998; Kindig & Poole, 1998; Kindig *et al.* 2002; Copp *et al.* 2009**). Moreover, a small proportion of capillaries may just support plasma flow whilst amongst others an order of magnitude difference in RBC flux, velocity and hematocrit is apparent and RBC capillary path-lengths vary substantially (**Sarelius & Duling, 1982; Poole *et al*. 1997; Kindig *et al*. 2002; rev. Poole *et al*. 2011**). However, certain established and recently-developed methods are predicated on the presumption that an individual capillary represents a singular unit of surface area, RBC flux and blood-myocyte exchange. Depending on the contractile activity of the muscle the small arterioles may assume a greater (rest) or lesser (exercise) role in blood-tissue O_2 flux (**Poole *et al.* 2011; Carvalho & Pittman, 2008; rev. Golub & Pittman, 2013**). Crucially, none of these complexities are readily apparent from bulk blood flow measurements across the body, limbs or discrete muscles; and yet they must play a commanding role in the efficacy by which the capillary bed subserves its major function(s). Moreover, because different transported molecules may rely preferentially upon RBCs (e.g., O_2) versus plasma (glucose, lactate, insulin) for transport descriptions of microvascular blood flow alone are inadequate to describe the potential for blood-myocyte exchange. Unfortunately it has become common practice, even in the peer-reviewed literature, to ignore or dismiss these considerations despite their overriding importance in understanding physiologic function.

Brief history of established viewpoint.

Following the work of Adolf Fick in the late nineteenth century, August and Marie Krogh's sentinel development and application of O_2 diffusion theory from the lung to skeletal muscle changed the manner in which scientists viewed capillary bed structure and function. In a series of papers that lead to his being awarded the 1920 Nobel prize in Physiology or Medicine, **Krogh** (**1919ab; 1920ab**) proposed that: A) Intramyocyte PO_2 decreased systematically with increasing distance from the nearest capillary (Krogh-Erlang model). This model calculated the presence of "anoxic loci" within which mitochondrial respiration ceased. B) In resting muscle most capillaries did not support RBC flux and were constricted or collapsed (no RBCs or plasma in their lumen). C) To support the increased metabolic demands of contractions and reduce diffusion distances to intramyocyte mitochondria more capillaries were "recruited" and hence supported RBC flux. These are the key facets of capillary function that are presented in most physiology textbooks and upon which many contemporary researchers interpret their findings (**Figure 1, left hand side**). An egregious omission from those utilizing Kroghian theory is due consideration of the conditions under which the data were collected and which Krogh himself regarded as a major limitation. Specifically, in order to successfully perfuse the muscles they remained for up to several days post-mortem before perfusion, leakage was stopped by tying off multiple vessels and extremely high (non-physiological) pressures were applied. India ink was used to identify which capillaries supported flow and the carbon particles tended to clump together at low perfusion rates limiting access especially to low-flow vessels. An important issue here is that, at higher flows, less carbon clumping would occur raising the possibility that during high flow conditions associated with exercise/contractions a more complete capillary bed filling would occur simply as a function of the experimental conditions and irrespective of whether an individual capillary has undergone

"recruitment" or not. Daniel J. Boorstin, the National Librarian of Congress, considered that "*The greatest impediment to scientific progress is not ignorance but the illusion of knowledge*" (**Boorstin, 1983**). It is tragic that Kroghian theory has become so entrenched that a wealth of experimental evidence to the contrary (see below) has often been ignored or otherwise dismissed. Indeed, the present situation regarding capillary function may be likened to the impediments to scientific discovery through the Middle-Ages where original observations were sometimes dismissed "*simply because they were not found in Aristotle.*"

Presumptions for which compelling contrary evidence has emerged since 1920.

Presumption: *A capillary "is a capillary is a capillary".* **Current perspective:** Across tissues capillaries may have widely divergent properties and the tacit presumption that capillary structure and function is equivalent across species and organs is specious. For example, capillaries in the mesentery and gut may have distinct pores and are thus highly porous to proteins and solutes (**Renkin & Tucker, 1998; rev. Poole *et al.* 2004**). By comparison the protein reflection coefficient of skeletal muscle is high indicating that the capillary endothelium presents a significant barrier to these and other molecules. In addition, capillaries in the lungs are poorly supported and collapse readily due to low intraluminal pressures and/or positive alveolar pressures, especially at the apices of the upright lung (**Pande & Hughes, 1983**). In marked contrast, capillaries in skeletal muscle are held open by collagenous struts that affix the capillary wall to the surrounding myocytes resisting collapse especially during muscle contraction (**Borg & Caulfield, 1980**).

Presumption: *Capillaries are straight unbranched structures.* **Current perspective:** Capillaries exhibit considerable tortuosity that changes dynamically as a function of muscle sarcomere length and contributes a significant additional length, volume and surface area above that calculated from the assumption of anisotropy (**Mathieu-Costello *et al.* 1989**). In addition, the capillary network may be highly branched with profuse intercapillary connections. Thus, rather than capillaries representing "pinpoint" O_2 sources it may be more appropriate to consider the Hill cylinder model which constitutes a far more efficient O_2 delivery system (**Ellis *et al.* 1983**).

Presumption: *A capillary represents a "quantum" unit of exchange that is unchanged from rest to contractions.* **Current perspective:** This is especially difficult to reconcile with the observations that capillaries display substantial variability in length e.g., 20-1,000 μm) and diameter (~2-8 μm) (**Mathieu-Costello *et al.* 1989**) and, in a given muscle at rest, inter-capillary RBC flux and velocity as well as hematocrit vary over an order of magnitude (**Poole *et al*. 1997; Kindig *et al.* 2002**). From rest to contractions capillary RBC flux, velocity and hematocrit as well as plasma flux all increase significantly (**Kindig *et al.* 2002**).

Presumption: *Pre-capillary sphincters control RBC and plasma flow through individual capillaries.* **Current perspective:** Evidence for the presence of functioning pre-capillary sphincters is weak (**rev. Golub & Pittman, 2013**). Conditions such as hyperoxia that stop flow to large regions of the capillary bed appear to do so via constriction of upstream arteriolar smooth muscle. There is no persuasive evidence that RBC flux can be stopped preferentially in a single capillary by active pre-capillary sphincter contraction (**Gorzynski & Duling, 1978; Gorczynski *et al.* 1978; Klitzman *et al.* 1982**).

Presumption: *The capillary endothelium is contractile and capillaries can therefore be "closed" and empty of RBCs and plasma. Most capillaries do not support RBC flux in resting muscle.* **Current perspective:** *In vivo* it would be extremely difficult to detect a collapsed capillary by light microscopy. However, observing the muscle capillary bed from control to pharmacologically maximally vasodilated conditions (**Kindig & Poole, 1998,1999,2001**) or from rest to contractions does not reveal the initiation of RBC flow in a significant proportion of previously non-flowing capillaries (**Kindig *et al.* 2002**).

***Presumption**: An increase in the number of RBC-flowing capillaries is necessary to support increased O_2 flux during muscle contractions.* **Current perspective:** By direct observation and measurements of RBC and O_2 flux the expected exponential profile of increased O_2 uptake is found in the absence of *de novo* capillary recruitment (**Behnke *et al.* 2001,2002**)

***Presumption:** O_2 diffusion distances from capillary to mitochondria limit mitochondrial O_2 delivery. Tissue PO_2 decreases proportionally with distance from the capillary.* **Current perspective:** Intramuscular measurements in dog gracilis frozen whilst contracting reveals that intramyocyte PO_2s are low and close to uniform (**Gayeski *et al.* 1987; rev. Honig *et al.* 1997; see also Voter & Gayeski, 1995** for a fuller consideration of technical limitations associated with the cryomicrospectrophotometrical technique utilized). The expected capillary-to-distant mitochondrial intramyocyte PO_2 gradients were absent. Proton magnetic resonance spectroscopy in human quadriceps during voluntary exercise confirms the presence of very low intramyocyte PO_2s (**Richardson *et al.* 1995)**.

***Presumption:** Structural capillary density is the major determinant of muscle O_2 diffusing capacity.* **Current perspective: Federspiel & Popel (1986)** and **Groebe & Thews (1990)** have developed theoretical models positing that the primary determinant of muscle O_2 diffusing capacity must be the number of RBCs in the capillaries adjacent to the contracting myocytes at a given instant. **Hepple *et al.* (2000)** completely dissociated muscle O_2 diffusing capacity from structural capillary density in the dog gastrocnemius-plantaris complex by comparison of immobilization-atrophied (↑capillary density, ↔O_2 diffusing capacity) with control and exercise-trained (↓capillary density caused by fibre hypertrophy, ↑O_2 diffusing capacity) conditions. Whereas Krogh considered the capillary and its catchment volume a discrete unit, exquisite measurements of O_2 flux and modeling studies within the microcirculation have revealed that there is diffusional O_2 transport among microvessels including arteriole to venule and capillary to capillary (**Ellsworth *et al*. 1994; Goldman & Popel, 2000; Goldman *et al*. 2006; Fraser *et al.* 2012**). Such fluxes may be proportionally more important in low blood flow/O_2 delivery conditions and the capillary-to-capillary interactions may serve to enhance the role of capillaries with low O_2 flux in supplying O_2 to more distal myocytes/regions. In addition, capillary tortuosity (**Mathieu-Costello *et al.* 1989; Poole & Mathieu-Costello, 1989**) significantly improves O_2 transport in healthy muscle as do capillary anastomoses especially when the proportion of non-RBC flowing capillaries increases, for example, in disease (**Goldman & Popel, 2000**).

Exemplar "contemporary" data supporting/opposing established (Kroghian) viewpoint and methodological concerns.

a. Supporting.

i. Analysis of capillaries recruited from histological sections. When viewed in cross-section, muscles preserved during contractions versus at rest evidence more RBC profiles in the capillary lumen. Concern. Both the presumption that presence of an RBC indicates RBC flux (i.e., that the capillary supported RBC flux) and that absence of an RBC is evidence that the capillary was not flowing are baseless. The appearance of more RBCs in capillary cross-sections from contracting muscle is to be expected based upon the demonstrated increase in capillary hematocrit as a function of contraction-induced elevation of capillary RBC flux and velocity (i.e., from ~10 up towards 45%) (**Klitzman & Duling, 1982; Kindig *et al.* 2002; Copp *et al.* 2009**).

ii. Reducing muscle PO_2 causes capillary recruitment. In resting cremaster muscle when the extant PO_2 is reduced from 130 to 35 mmHg a robust increase in the density of capillaries was visualized by fluorescently-labelled plasma (**Parsatharathi & Lipowsky, 1999**). Concern. Careful attention to physiological conditions is

important to interpreting these data. Yes, hyperoxia is a potent vasoconstrictor and hypoxia a vasodilator. The authors rightly considered that 130 mmHg PO_2 is hyperoxic: Indeed, this PO_2 is generally only experienced by organs in immediate contact with the atmospheric air (e.g., skin, corneal surface, mucous membranes etc.). However, it is what they considered to be the "low PO_2" of 35 mmHg that is at issue here. Direct measurements of resting "muscle" PO_2 place it ~17 mmHg (**Whalen *et al.* 1974, 1976**) indicating that **Parsatharathi & Lipowsky (1999)** were actually observing a reversal of hyperoxic vasoconstriction rather than (as they considered) a hypoxic capillary recruitment that they predicted based upon Krogh's theory. This specific (and, unfortunately, all too common) instance exposes the dangers of presuming, rather than measuring, PO_2 within discrete compartments. Unless PO_2 is measured the designations of normoxic, hypoxic and hyperoxic (and any supposedly associated behavior) cannot, and therefore, should not, be made.

iii. Intravital microscopy reports of a high proportion of capillaries in resting skeletal muscle that do not flow. In addition to the majority of research papers that simply presume that most capillaries do not support RBC flux in resting muscle (see caustic opinion of lauded microcirculationist Professor Eugene Renkin (**Renkin, 2002**) directed to **Dr. Bentzer and colleagues (2001)** regarding this practice) there are intravital microscopy observations supporting this notion (e.g., **Gorcynski & Duling, 1978; Gray *et al.* 1983; Hargreaves *et al.* 1990**). Concern. Capillaries are perilously fragile structures whose ability to support RBC flux may be imperiled by multiple conditions imposed by experimental constraints. For instance: a. Systemic hypotension consequent to anesthesia and/or systemic hypoxia acts to reduce muscle blood flow mechanically via reduced driving pressure and also in response to a sympathetically-mediated arteriolar vasoconstrictive tone invoked to constrain the falling blood pressure. This concern underscores the necessity of measuring and reporting arterial blood pressures continuously with intravital microscopy observations. b. Mechanical damage incurred during surgery necessary to expose or exteriorize muscle can stop capillaries flowing. c. Stretched muscles become thinner and more translucent enabling clearer imaging of myocytes and capillaries by transmission light microscopy. However, increasing sarcomere length much beyond ~3 µm stretches the capillary and narrows its luminal diameter increasing resistance to flow and also induces a reflex arteriolar vasoconstriction decreasing RBC flux through the affected capillary units (**Welsh & Segal, 1996; rev. Kindig & Poole, 2001**). To date, we are unaware of any preparations that have documented a high proportion of non-RBC flowing capillaries at measured sarcomere lengths $\leq$ 3 µm in healthy normotensive and normoxemic animals.

iv. Indirect methods. 1-methyl xanthine (1-MX). Based on the premise that xanthine oxidase is located primarily in the capillary endothelium, Clark and colleagues (**Rattigan *et al.* 1997ab; Clark *et al.* 1998; rev. Clark *et al.* 2008**) considered that the arterial-venous difference of 1-MX was quantitatively synonymous with capillary recruitment across different metabolic conditions (insulin infusion, contractions) in the rat hindlimb. Concerns. For this technique to detect or measure capillary recruitment it must be demonstrated that 1-MX metabolism increases in direct proportion to the number of RBC-perfused capillaries: despite 1-MX being plasma-borne. Also, 1-MX metabolism must be independent of the rate of plasma (or RBC) flow through capillaries (i.e., capillary hemodynamics). Recall that practically all capillaries support at least plasma flow in the rat at any given time (**Kayar & Banchero, 1985; Snyder *et al.* 1992**) and thus, it is likely that the increased 1-MX metabolism measured using this method reflects flow-weighted 1-MX metabolism within capillaries that supported (at least) plasma flow under control conditions. Additional challenges to the practicality of this technique are that, in addition to the capillary endothelium, xanthine oxidase is found in smooth muscle and plasma (**Hellsten-**

Westing, 1993; Newaz & Adeeb, 1998). In fact, xanthine oxidase activity in the intact rat hindlimb is so great that the animal must be pretreated with allopurinol (a xanthine oxidase inhibitor) to prevent all infused 1-MX being metabolized under control conditions. This method lacks any gold-standard comparisons and has not been tested in concert with visualization of capillary hemodynamics. Contrast-enhanced Ultrasound (CEU). CEU possesses the significant advantage that it can be used to assess human muscle under resting and voluntary exercise conditions. CEU entails interpreting an acoustic signal derived from the distribution of inert gas microbubbles infused intravenously. These bubbles are rigid spheres that are significantly smaller than RBCs and considered to "*track with erythrocytes through the vasculature*" (**Clark *et al.* 2008**) whilst not changing blood flow rheology. Concerns. At this time we are unaware that these notions have been tested empirically. Crucially, as a formal characterization of RBC distribution in the capillary bed from rest-to-exercise has only been made for the rat spinotrapezius muscle claims that CEU bubbles distribute in proportion to RBCs in the capillaries cannot be validated. However, even if this is true, the increases in bubble concentration caused by insulin and/or contractions would be expected on the basis of increased capillary hematocrit within already flowing vessels and would not, therefore, be *prima facie* evidence for *de novo* capillary recruitment. Moreover, as capillary hematocrit increases (and thus the proportion of the vessels occupied by plasma decreases) the distribution volume of the plasma-borne bubbles would decrease; further complicating interpretation of vascular volume changes from bubble "density." Moreover, because the microbubble concentration is far less than that of RBCs, their flow distribution from arteriolar to arteriolar bifurcation cannot be equated with RBC distribution, in fact it more likely reflects plasma distribution (as do platelets). Vascular volume estimates from microbubbles only represent the vascular volume traversed by bubbles under those set of conditions. A flow redistribution that impacts plasma and microbubbles behavior at arteriolar bifurcations would appear to cause alterations in vascular volume, i.e., false appearance of capillary recruitment. Despite these concerns, this technique has become the standard approach for assessing capillary recruitment in human muscle in response to insulin infusions and muscle contractions (e.g., **Meijer *et al.* 2002; Wang *et al.* 2013**). Permeability Surface Area Product (PSA). This variable is often measured using glucose extraction and presumes that increased extraction or fractional extraction can only occur via recruitment of additional microvascular surface area (i.e., *de novo* plasma flow in newly recruited capillaries, e.g., **Jansson et al. 2010; Murdolo et al. 2008, 2013**). Concerns. In addition to this presumption, the PSA (glucose) approach for measuring capillary recruitment neglects fundamental principles of the physiological regulation of blood-myocyte glucose flux. Specifically, as reviewed by **Wasserman & colleagues (2011)** blood-myocyte glucose flux follows a distributed control paradigm incorporating not only vascular glucose delivery but also GLUT4 transport of glucose into the myocytes and subsequent phosphorylation by hexokinase. Thus, without knowing the impact of the experimental manipulation on GLUT 4 and hexokinase function little can be concluded about changes in microvascular surface area *per se*.

b. Opposing

The compelling weight of evidence across a spectrum of experimental methodologies utilizing anesthetized animals up to voluntarily exercising human muscles supports that little *de novo* capillary recruitment is likely (or indeed necessary) to support increased blood-myocyte exchange from rest to exercise.

i. Most capillaries support RBC flux in resting muscle and therefore are not available to be recruited. In muscles ranging widely in fibre-type composition and oxidative capacity from the cat sartorius (**Burton&**

Johnson, 1972), rabbit tenuissimus (**Oude Vrielink *et al.* 1987**) to hamster cremaster and sartorius (**Damon & Duling, 1984**) and the rat spinotrapezius, diaphragm (**Kindig & Poole, 1998,2001; Kindig *et al.* 1999,2002**) and extensor digitorum longus (**Anderson *et al.* 1997; Ellis *et al.* 2002**) most capillaries support RBC flux at rest.

ii. In conscious and anesthetized animals almost all muscle capillaries support plasma flow. Infusion of plasma and capillary endothelial markers (e.g., Thioflavin-S) identifies the presence of flow (could not discriminate plasma from RBCs) in essentially all capillaries within seconds of infusion in skeletal muscle (**Kayar & Banchero, 1985; Snyder *et al.* 1992**) and heart (**Vetterlein *et al.* 1982**). Thus, these capillaries cannot be "closed."

iii. Anesthesia and surgical exposure/exteriorization do not impair arteriolar smooth muscle function. Radiolabelled microsphere determination of bulk muscle blood flow demonstrates that surgery and anesthesia necessary for exteriorization of the muscles necessary for intravital microscopy observation does not systematically affect spinotrapezius blood flow (**Bailey *et al.* 2000**). Moreover, across metabolic transients evoked by electrically-induced muscle contractions these procedures do not alter the relationship (~6:1 L/L) between blood flow and $\dot{V}O_2$ (**Ferreira *et al.* 2006**).

iv. Measurements of blood-myocyte O_2 flux in capillaries indicate that *de novo* capillary recruitment is not requisite to support $\dot{V}O_2$ kinetics. Measurements of pulmonary (alveolar) and leg muscle(s) gas exchange in humans reveal a time constant (τ) for the primary $\dot{V}O_2$ component (Phase II) of 20-30 s in healthy young subjects following the onset of moderate intensity exercise (**rev. Poole & Jones, 2012**). The rat spinotrapezius has a similar oxidative enzyme capacity (citrate synthase) as the human quadriceps and would therefore be expected to, and in fact does, display similar $\dot{V}O_2$ kinetics following the onset of electrically-induced muscle contractions (**Behnke *et al.* 2002**) despite the absence of any detectable *de novo* capillary recruitment (**Kindig *et al.* 2002**) and in the presence of the surgical interventions and anesthesia required for the intravital microscopy preparation.

v. Near infrared spectroscopy (NIRS) measurements of changes in muscle [hemoglobin] are inconsistent with substantial *de novo* capillary recruitment. Kroghian theory wherein the vast majority (up to 90 or more per cent) of capillaries move from RBC-absent to RBC-flowing status when they are recruited predicts a substantial (≥9 fold) increase in muscle [hemoglobin]. In contrast, in the absence of *de novo* capillary recruitment (based upon the evidence presented above), it would be hypothesized that muscle [hemoglobin] in the exercising human quadriceps would not, and indeed, could not, increase substantially (i.e., many fold) above that found at rest: any increases being reliant upon elevated hematocrit within already RBC flowing capillaries. NIRS detects the total concentration of hemoglobin and myoglobin (oxygenated and deoxygenated forms). Following the rationale that [myoglobin] in the volume sampled will not change from rest to contractions any change in the [hemoglobin+myoglobin] must arise from [hemoglobin], Barstow and colleagues (**Lutjemeier *et al.* 2008; Davis & Barstow, 2013**) determined that, from rest to moderate, heavy or severe-intensity cycling exercise quadriceps [hemoglobin] increased up to 30%. This magnitude of increase can readily be accounted for by the elevation of capillary hematocrit found at higher flow rates in the absence of *de novo* capillary recruitment.

Problems of interpretation at the microcirculation-physiology interface.

As we have seen, acceptance of the Krogh model leads to untenable and experimentally falsifiable predictions. In addition to those addressed previously at least four more of these falsifiable predictions are immediately pertinent to understanding how muscle O_2 diffusing capacity increases (up to two orders of magnitude) from rest-contractions and why, at least in health at rest and at submaximal exercising metabolic rates (i.e., < $\dot{V}O_2$max), the site of control of oxidative metabolism resides at the mitochondrial level rather than proximally in the O_2 transport pathway.

1. *Adherence to Kroghian theory has led to the notion that a capillary represents a quantum unit of exchange that is present when that capillary is flowing (plasma and/or RBCs depending on substrate) and absent when it is not.* For O_2 this notion contravenes the physics of O_2 transport wherein its low solubility (0.003 ml/100 ml/mmHg) dictates that very little O_2 is dissolved in plasma and hence its diffusing capacity for O_2 is extremely low. Thus, the diffusing capacity for O_2 and therefore the blood-myocyte O_2 flux potential will depend upon the number of RBCs within the capillary at any given time. Thus the surface area of the capillary viable for blood-myocyte O_2 flux will be a function of the capillary length and hematocrit. Notwithstanding the extraordinary heterogeneity of capillary lengths and diameters mentioned above, inspection of resting or contracting muscles reveals capillaries with hematocrits, RBC fluxes and RBC velocities ranging over more than an order of magnitude and changing as a function of time within a given capillary. It is impossible to reconcile that each of these capillaries supports the same O_2 flux or that, when RBC flux and hematocrit increase substantially from rest-contractions, that O_2 flux does not increase in any given capillary. The same argument may be made for increased plasma flow for glucose, free fatty acids and hormonal transcapillary flux/action. Direct inspection by intravital microscopy reveals that, from rest to contractions, the sentinel changes (in order of proportional increases) are in capillary RBC flux, velocity and capillary hematocrit (**Hudlicka *et al.* 1982; Klitzman *et al.* 1982; Hargreaves *et al.* 1990; Kindig *et al.* 2002; rev. Poole *et al.* 2011; Poole & Jones, 2012**).
2. *In a system where capillary recruitment is matched to muscle metabolic rate ($\dot{V}O_2$) fractional O_2 extraction would be high at rest and not increase as a function of $\dot{V}O_2$.* Across a multitude of muscles and muscle groups and the whole body in all species investigated by direct measurements and also NIRS and phosphorescence quenching fractional O_2 extraction increases as a hyperbolic function of $\dot{V}O_2$ (e.g., **Grassi *et al.* 1996; Ferreira *et al.* 2006; rev. Poole & Jones, 2012**).
3. *At rest and during submaximal contractions a widely-held notion is that muscle $\dot{V}O_2$ and mitochondrial ATP production is controlled by the O_2 transport system.* For large muscle mass exercise $\dot{V}O_2$max is limited by the differential capacities of the lungs, heart and skeletal muscle for perfusive and diffusive O_2 transport (**Wagner *et al.* 1997**). In marked contrast, in healthy muscle at lower metabolic rates (rest, submaximal exercise) mitochondrial ATP production and thus $\dot{V}O_2$ control resides in the high energy phosphate signaling pathway (**Meyer & Foley, 1996; Poole & Jones, 2012**). As demonstrated by **Grassi *et al.* (1996)** in voluntarily exercising humans and **Behnke *et al. (*2001,2002)** in the rat spinotrapezius muscle, following the onset of contractions increased muscle O_2 delivery matches or exceeds O_2 demands ($\dot{V}O_2$), consequent to rapid vasodilation (**Poole *et al.* 2007; Behnke & Delp, 2010**) and muscle pump activity, such that microvascular PO_2 does not plummet but remains constant for 10-20 s prior to falling in a close-to-exponential fashion to reflect the increased fractional O_2 extraction.
4. *Observation of muscle microcirculation in skeletal muscles of animals suffering from chronic heart failure (CHF), Type I and II diabetes as well as sepsis reveals the absence of capillary RBC flux in up to 50% or more of the capillaries with many vessels that are flowing exhibiting low RBC velocity and sporadic flow* (**Lam *et al.* 1994;**

Bateman *et al*. 2001; Ellis *et al.* 2002; Richardson *et al.* 2003; Kindig *et al.* 1998, 1999; Padilla *et al.* 2006). Without knowing what the healthy muscle microcirculation looks like the capacity to identify pathological changes is abrogated. This situation erodes the capacity to understand the mechanistic bases for O_2 transport deficits in CHF and diabetes. That access to a significant portion of the body's total capillary surface area becomes compromised would, in-and-of itself, be expected to increase insulin resistance and impair glucose tolerance. Thus, recognition that capillary hemodynamics are deficient in these conditions is not possible from adherence to the Kroghian model; and yet is key to targeting development of therapeutic interventions designed to redress the actual mechanisms of dysfunction.

Revised (new) model of capillary function.

If increased blood-myocyte O_2 and substrate flux during contractions is inexplicable based upon established capillary recruitment theory how is it possible to account mechanistically for the up-to-100 fold increases in muscle perfusive and diffusive O_2 conductances across the physiological range (i.e., rest-maximal exercise)? At least five elements (four supported by empirical evidence and one, tentative i.e., "E") must be considered here:

A. In muscle at rest a large proportion of capillaries exhibit low RBC flux and velocity and, hence, cannot contribute substantially to overall blood-myocyte O_2 flux (**Burton & Johnson, 1972; Eriksson & Myrhage, 1972; Renkin *et al.* 1981; Hudlicka *et al.* 1982; Vetterlein *et al.* 1982; Damon & Duling, 1982; Kayar & Banchero, 1985; Dawson *et al.* 1987; Poole *et al.* 1997; Kindig & Poole, 1998,2001; Ellis *et al.* 2002; Kindig *et al.* 1997,2002; Russell *et al.* 2003; Richardson *et al.* 2003; Padilla *et al.* 2006; Copp *et al.* 2009**). During contractions these vessels increase their RBC flux disproportionally to the mean capillary hemodynamic response and become quantitatively more important to the overall muscle $\dot{V}O_2$ (**Hargreaves *et al.* 1990; Kindig *et al.* 2002; Brown *et al.* 2005**). Great importance has been placed upon the value of *de novo* capillary recruitment serving to limit the fall in capillary RBC transit times during exercise. However, if, as the data support, most capillaries support RBC flux at rest this condition serves to maximize RBC (and also plasma) transit time. During exercise capillary RBC transit time will fall to exactly the same point that it would have supposing capillary recruitment and it has been demonstrated that even disappearingly small (~0.1 s) mean transit times, present at blood flows ~4L/kg/min, do not appear to constrain RBC O_2 offloading (**Richardson *et al.* 1993, 1994**).
B. Despite traversing the entire capillary length in resting muscle, the relatively high intramyocyte PO_2 and low fractional O_2 extraction (i.e., 0.25-0.50) may restrict the capillary distance over which O_2 flux occurs such that relatively little of the capillary surface area available for blood-myocyte O_2 transfer is utilized. When RBC velocity increases during contractions and intramyocyte PO_2 falls, the linear distance along the capillary used for the increased fractional O_2 extraction (to ~0.9) will increase. This process has been termed "longitudinal recruitment" and may substantially increase the capillary surface area participating in blood-myocyte O_2 diffusion (**Poole *et al.* 2011**).
C. The very low mean capillary hematocrit present in resting muscle (~15%) increases during contractions towards systemic values (~45%). Thus, in the complete absence of *de novo* RBC flux in capillaries this may cause a substantial increase in capillary RBC content (and muscle [hemoglobin]) and hence O_2 diffusing capacity.
D. The PO_2 gradient between capillary and myocyte is preserved by the presence of a positive intercept and the maintenance of an ~6:1 ratio of the blood flow-$\dot{V}O_2$ relation across the range of achievable metabolic rates

(**Ferreira *et al.* 2006; Poole & Jones, 2012**) combined with the low intracellular PO_2 (**Richardson et al. 1995; Honig et al. 1997**).

E. The capillary endothelial surface layer is fundamental to setting the capillary hematocrit (**Desjardins & Duling, 1990**) and likely capillary hemodynamics themselves; though the effect of RBC distribution at arteriolar bifurcations should not be neglected in this respect (**Elsworth et al. 2009**). The extent to which the endothelial layer impacts the physiological and pathophysiological (**Zuurbier *et al.* 2005; Lemkes *et al.* 2012; Torres-Filho *et al.* 2013**) behavior within the microcirculation remains to be elucidated.

Re-evaluation of current data

As dealt with above (see methodological concerns iv above), the contemporary scientific literature is replete with indirect measurements (primarily 1-MX, CEU, PSA (glucose)) claiming to measure microvascular blood volume and attributing experimental increases of such to capillary recruitment (e.g., **Wheatley *et al.* 2004; Meijer *et al.* 2012; Chadderdon *et al.* 2012; Murdolo *et al.* 2013; Wang *et al.* 2013**). Unfortunately, these approaches are each assumption-laden such that the resultant data and their interpretation are likely far removed from the actual events in the microcirculation. One example of this is the extraordinary values for capillary blood volume in the exercising forearm muscles of rhesus macaques (Macaca mulatta) derived by CEU. The 20% (0.2 ml/g) capillary volume per muscle volume exceeds that in humans by 5-10 fold (**Richardson *et al.* 1994**) such that it approaches that found in mammalian heart muscle and was unsupported by rigorous morphometric analysis of the muscle. Moreover, as an exemplar of the over-riding concerns of reverting to 1920s microcirculatory perspectives the recent paper of Meijer et al. (2012) is relevant. In recognition of the serious criticisms leveled at CEU (e.g., **Poole *et al.* 2008**) **Meijer & colleagues (2012)**, to their credit, have compared CEU in non-glabrous skin (nailfold) to muscle with respect to insulin-mediated actions. The nailfold facilitates direct observation of human skin capillaries without the necessity for anesthesia or surgical intervention. Despite that their paper is entitled "*Insulin-induced microvascular recruitment in skin and muscle are related*…" and that the fundamental functions and control of blood flow in non-glabrous skin and muscle are different, the contention of **Meijer *et al.* (2012)** is that nailfold skin offers a window into skeletal muscle microcirculatory function and dysfunction. Inspection of their data, however, indicates otherwise. Specifically, in their Figure 4 CEU-measured changes in insulin-mediated muscle blood volume of up to 250% cannot quantitatively result from the ~50% increase in capillary recruitment as measured by microscopy in nailfold. Any mechanistic association ($r=0.57$, $p<0.02$) or "parallel" behavior touted between these variables is further brought into question by the fact that, for a CEU-measured increase of muscle blood volume of 100% skin capillary recruitment varies from 4-50% (i.e., over 12-fold) and, at an insulin-augmented capillary recruitment of ~10%, muscle blood volume changes purportedly range from -50 up to 175%. If muscle and skin are indeed behaving similarly (though there is no solid evidence that they do in Meijer et al. or indeed should do) capillary or microvascular recruitment cannot explain the changes in muscle blood volume claimed on the basis of CEU measurements. It is our contention that peer-acceptance of such data and subsequent questionable conclusions is made possible, or at least facilitated, by adherence to a dated theoretical framework of capillary structure and function rather than the wealth of subsequent empirical microcirculatory observations and contemporary models emerging therefrom as presented herein.

Conclusions

The nine decades since Krogh presented his theories of muscle capillary function and blood-myocyte O_2 flux have seen those theories tested rigorously. Facilitated, in part, via technical advancement and elegant experimental designs key elements of those theories have proven untenable. Our understanding of vascular control and the dynamics of blood flow, capillary microscopic structure (e.g., tortuosity/branching, endothelial surface layer) arteriolar vasomotor control, capillary hemodynamics and O_2 flux has advanced exponentially (Figure 1). By direct observation (Krogh's favoured approach) of RBC flux in capillaries and supported by measurements of microvascular and intramyocyte PO_2 and *in vivo* approaches key aspects of muscle microvascular function have been revised. These revisions are presented graphically in Figure1 and include: 1. The majority of capillaries support RBC flux at rest and thus are not available to be recruited during exercise. During contractions the $\dot{V}O_2$ kinetics response can occur in the absence of *de novo* capillary recruitment. Thus, increased RBC flux and velocity occur within already flowing capillaries. 2. There is a substantial range of capillary RBC flux, velocity and hematocrit in resting and contracting muscle such that not all capillaries are equally important with regard to O_2 and substrate delivery. Crucially, capillary hematocrit at rest is only ~33% of systemic. 3. Capillary RBC flux and velocity increase within the first contraction cycle (at 1 Hz) long before microvascular PO_2 decreases supporting a rapid "feed-forward" vasodilation that matches or precedes increased mitochondrial O_2 demands (**Behnke *et al*. 2001,2002; Poole *et al.* 2007; Behnke & Delp, 2010**). 4. Key mechanisms responsible for the exercise-induced increase in muscle O_2 diffusing capacity are recruitment of additional capillary surface for exchange along the length of capillaries (i.e., longitudinal recruitment), increased proportion of capillaries that contribute substantially to blood-myocyte flux, elevated capillary hematocrit that increases the number of RBCs per unit capillary length and thus diffusing capacity and a reduction in intramyocyte myoglobin saturation that reduces the so-called "O_2 carrier-free region" facilitating enhanced intramyocyte O_2 flux (**Honig *et al.* 1997**). 5. The greatest PO_2 drop occurs in close proximity to the capillary such that, in contracting myocytes PO_2 is low and, within the limits of current technology, PO_2 gradients are undetectable (**Honig *et al.* 1997; Richardson *et al.* 1995**). This suggests that events in the capillary bed (i.e., number of RBCs in capillaries adjacent to myocytes, their spacing and transit time) constitute a primary determinant of muscle O_2 diffusing capacity and undermines the simple Kroghian concept of O_2 diffusion distances (**see Federspiel & Popel, 1986; Groebe & Thews, 1990; Goldman *et al.* 2006**). 6. Looking forward, investigation of the role of the endothelial surface layer in setting capillary hemodynamics in health and disease represents a particularly exciting contemporary field of investigation (e.g., **Zuurbier *et al.* 2005; Torres-Filho *et al.* 2013, rev. Lemkes *et al.* 2012**).

This review issues a plea for present day researchers not to ignore the substantial progress that has been made since 1920 in understanding muscle microcirculatory function. In the face of compelling evidence to the contrary, rigid adherence to Krogh's theories goes against basic scientific principles, and the very ethos of that extraordinary scientist.

"Whereof one cannot speak, thereof one must be silent." Wittgenstein (1922).

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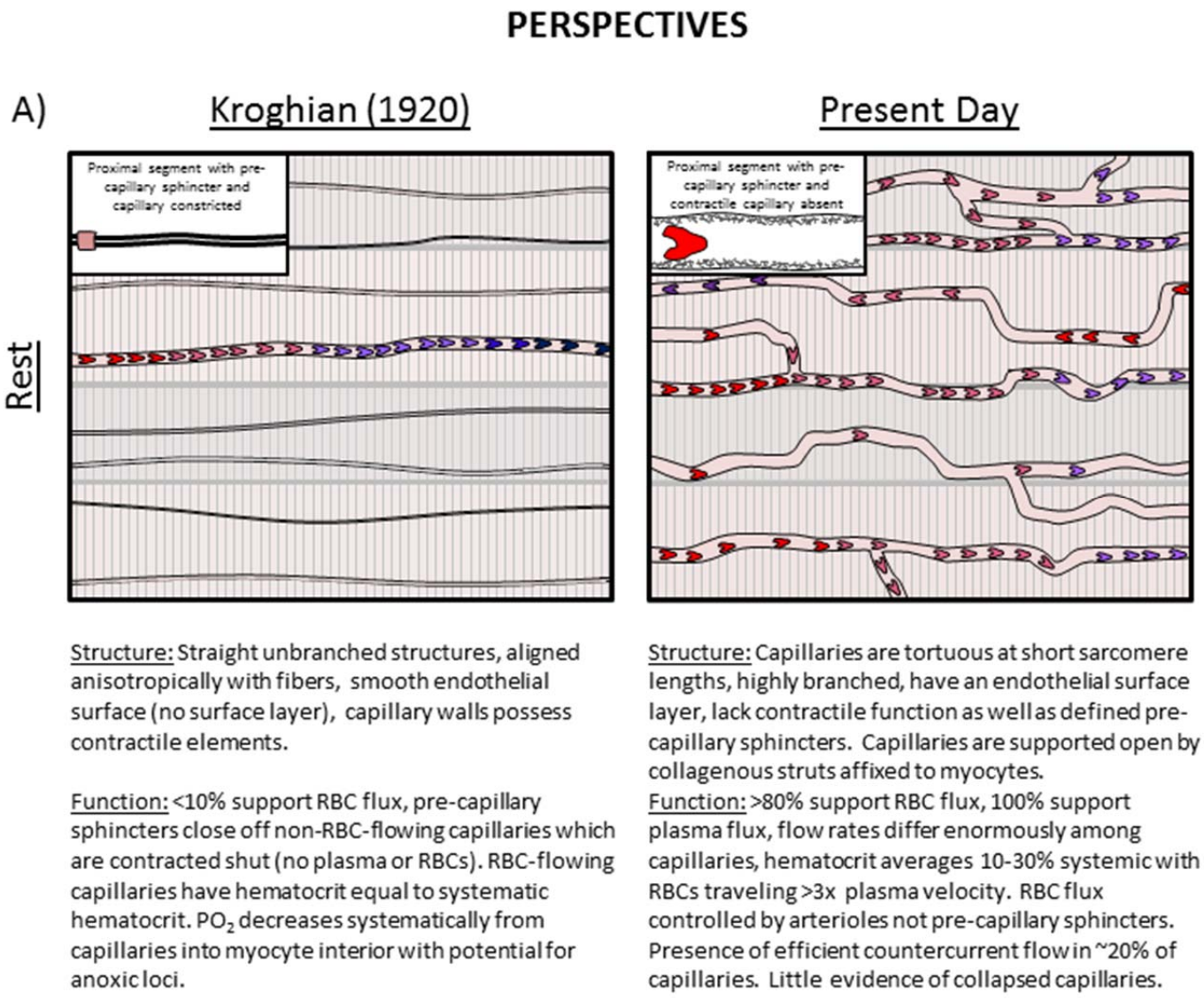
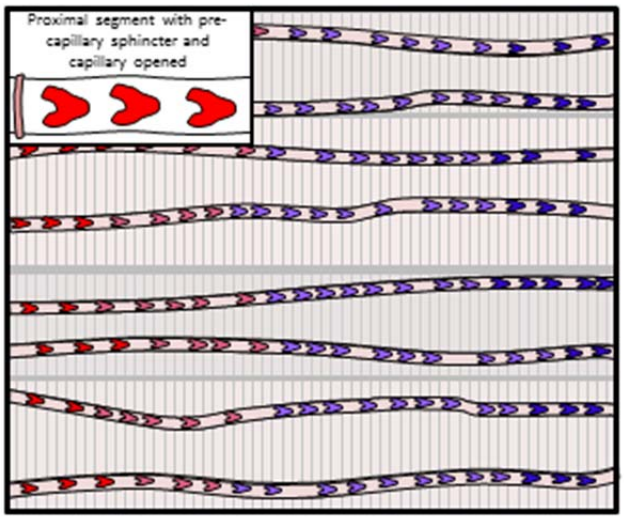
Figure

Figure 1. From 1920 to the present day scientific discoveries have revolutionized our understanding of skeletal muscle capillary structure and function.

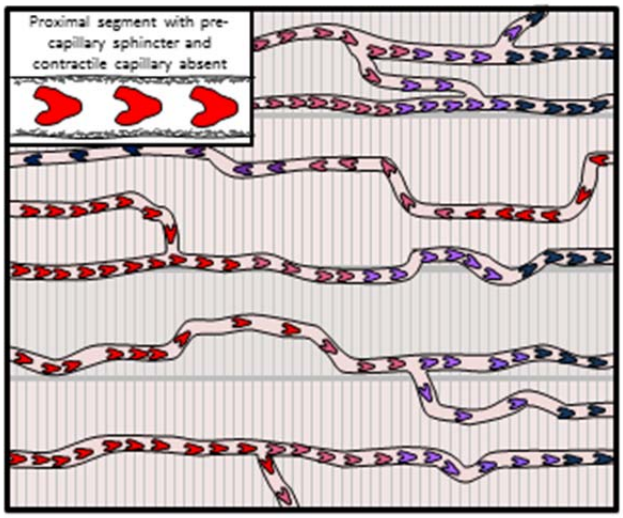


B)

Exercise



Essentially all capillaries support RBC flux. In response to low PO_2, metabolites produce vasodilation & open up arterioles & pre-capillary sphincters. Diffusion distances are reduced but possibility of anoxic loci considered.



Little/no initiation of RBC flux in previously non-flowing capillaries. RBC flux and velocity increase substantially raising the number of capillaries important for O_2 & substrate delivery. This occurs in first contraction cycle (~1 s) prior to metabolite build-up. Capillary hematocrit increases toward systemic values elevating number of RBCs adjacent to myocytes & O_2 diffusing capacity. **Capillary surface area recruited along the length of RBC flowing capillaries.** Massive heterogeneity of capillary RBC flux, velocity & hematocrit remains despite many-fold increase in overall RBC flux. Sympathetic nervous system (constriction) & nitric oxide (dilation) exert commanding influence on total RBC flux – though system considered extremely complex with multitude of control redundancy. At contractions onset O_2 delivery increases so rapidly that microvascular PO_2 remains constant or increases for 10-20 s before falling to steady-state value determined by fractional O_2 extraction. Intramyocyte PO_2 falls to ~1-3 mmHg evidencing no detectable gradients rendering importance/relevance of O_2 diffusion distances & presence of anoxic loci questionable. Microvascular PO_2 is fiber-type dependent & much lower in Type II (fast twitch) muscles.