LOCAL CAPILLARY SUPPLY IN MUSCLE IS NOT DETERMINED BY LOCAL OXIDATIVE CAPACITY

Alessandra Bosutti[1,2], *Stuart Egginton*[3], *Yoann Barnouin*[2], *Bergita Ganse*[4], *Jörn Rittweger*[4], *Hans Degens*[2,*]

[1]Department of Medical, Surgery and Health Sciences, University of Trieste, Trieste, Italy; [2]School of Healthcare Science, Manchester Metropolitan University, United Kingdom; [3]School of Biomedical Sciences, University of Leeds, United Kingdom; [4]Institute of Aerospace Medicine, German Aerospace Centre, Linder Höhe, 51147 Cologne, Germany

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*Address for correspondence:

School of Healthcare Science
Manchester Metropolitan University
John Dalton Building; Chester Street
Manchester M1 5GD
United Kingdom

Phone: +44.161.247.5686
E-mail: h.degens@mmu.ac.uk

Summary

We provide evidence that the maximal oxygen demand from surrounding fibres differs greatly between individual capillaries of human muscle. This observation may require a fundamental review of determinants of muscle capillarisation.

Abbreviations

DAF: Domains supplying a fibre

CAF: Capillaries around a fibre

FCSA: Fibre cross-sectional area

LCFR: Local capillary to fibre ratio

MO_{2max}: maximal oxygen consumption

SDH: Succinate dehydrogenase

VL: Vastus lateralis muscle

V_{mito}: total mitochondrial volume

Abstract

It is thought that the prime determinant of global muscle capillary density is the mean oxidative capacity. However, feedback control during maturational growth or adaptive remodelling of local muscle capillarisation is likely more complex than simply matching O_2 supply and demand in response to integrated tissue function. We tested the hypothesis that the maximal oxygen consumption (MO_2max) supported by a capillary is relatively constant, and independent of the volume of tissue supplied (capillary domain). We demonstrate that local MO_2max assessed by succinate dehydrogenase histochemistry 1) varied more than 100-fold between individual capillaries and 2) was positively correlated to capillary domain area in both human vastus lateralis ($R=0.750$, $P<0.001$) and soleus ($R=0.697$, $P<0.001$) muscles. This suggests that, in contrast to common assumptions, capillarisation is not primarily dictated by local oxidative capacity, but rather by factors such as fibre size, or consequences of differences in fibre size such as substrate delivery/metabolite removal.

Introduction

Highly oxidative muscles have a denser capillary network than those with a high glycolytic capacity, and within a given muscle, e.g. rat plantaris, fibres in the oxidative region have a higher capillary density than those in the more glycolytic region (Wust et al., 2009). This correlation between anatomical capillary supply and tissue oxidative capacity also seems to apply at a smaller scale of biological control, where the local capillary supply to an individual fibre appears to be positively related to its oxidative capacity (Bekedam et al., 2003). Importantly, in such studies, fibre size was not considered and hence the influence of local diffusion distances on cellular oxygenation cannot be assessed. We have previously demonstrated that the local capillary supply to a fibre correlates with its cross-sectional area and is only slightly modulated by its oxidative capacity, but not by phenotype (Egginton and Gaffney, 2010; Wust et al., 2009). However, if the principle of symmorphosis, that states structures are matched to functional demand, is valid then local capillarisation in a muscle should be arranged so that maximal oxygen demand per capillary is tightly regulated. To explore whether local feedback results in each capillary serving a similar maximal demand for oxygen, we estimated the supply areas (domains) of individual capillaries (Al-Shammari et al., 2014). Capillary domains provide a good estimate of the tissue oxygenation capacity of a capillary, even in muscles containing a mixture of fibres with different metabolic demand

(Al-Shammari et al., 2014), while the total volume of mitochondria, as reflected by succinate dehydrogenase activity, in a domain is a reflection of the maximal oxygen demand served by that capillary. We hypothesised that if the primary determinant of local capillary supply was local oxygen demand, then the maximal oxygen demand (MO_{2max}) supported by each capillary should be similar for each capillary.

Materials and Methods

Human muscle biopsy

Muscle biopsies were aseptically taken with a Rongeur forceps (Zepf Medizintechnik, Germany) under local anaesthesia (2 mL 1% lidocaine s.c.) from the vastus lateralis (VL) and soleus (SOL) of young men (*n*=10; 23-43 years old), following local ethical approval by the independent ethics committee Ärztekammer Nordrhein, Düsseldorf, Germany (No. 2010426) and written informed consent. The biopsies were taken as part of a bed rest study (ClinicalTrials.gov registration number NCT01655979) using baseline samples only. To facilitate longitudinal orientation, samples were embedded in a silicone tube filled with Optimal Cutting Temperature compound (Scigen® Gardena), frozen in liquid nitrogen and stored at -80°C until analysis. All participants underwent an extensive health screening.

Morphometry

Serial frozen sections (8 µm) were co-stained with biotinylated lectin (*Ulex europaeus* agglutinin I, Vector Laboratories, UK; 1 h, 50 $\mu L \cdot mL^{-1}$ in 1% BSA HEPES) and anti-mouse myosin type I (1:100; Novocastra, Leica Biosystems, UK; Product code: NCL-MHCs) to reveal capillary locations and Type I fibres, respectively. Sections were subsequently incubated with a secondary goat anti-mouse horseradish peroxidase labelled antibody (30 min, 1:200; Dako, UK) and stained (Vector® VIP HRP substrate kit), as described by the manufacturer. The sections were mounted in glycerol-gelatine and stored at 4°C. Serial sections were stained for succinate dehydrogenase (SDH) activity as described previously (Wust et al., 2009). Briefly, sections were incubated in the dark (20 min, 37 ^{0}C in 37 mM phosphate buffer, 74 mM sodium acetate and 0.4 mM tetranitroblue tetrazolium, pH 7.6) (Fig. 1A,B). The optical density at 660 nm (OD_{660}) determined (ImageJ; National Institute of Health, Bethesda, USA) as an index of the aerobic capacity/mitochondrial content of muscle

fibres since OD_{660} is linearly related to fibre MO_2max (Wust et al., 2009). For each image, a separate calibration curve was constructed with a series of filters with a known OD_{660} to remove potential optical bias related to differences in background intensity and lighting between sections.

Coordinates of fibre outlines and capillary centres (Fig. 1C) were collected using a digitising tablet (MMII 1201, Summagraphics Digitizers, Austin, Texas, USA) and analysed (AnaTis, BaLoH Software, http://www.baloh.nl) to calculate capillary domains (Fig. 1C) and parameters related to muscle fibre size (Wust et al., 2009). A capillary domain was defined as the area of tissue closer to a given capillary than neighbouring capillaries, which is a good estimate of capillary oxygen supply areas in muscles with heterogeneous fibre composition (Al-Shammari et al., 2014). Capillary domains overlap with portions of different fibres surrounding the vessel, and their combined maximal oxygen demand (MO_2max), obtained from the same fibres in a serial section stained for SDH, was calculated as:

$$MO_2max = \Sigma\ (SDH\ OD * Aovl)$$

where 'SDH OD' is the SDH OD for an overlap (domain) area, and 'Aovl' is the area of the overlap of the domain with an individual fibre (Fig. 1Ci-Ciii). The MO_2max was calculated for an average of 160 capillary domains per muscle and individual. Local capillary to fibre ratio (LCFR) is the sum of domain fractions overlapping a given fibre (Fig. 1Ci-Ciii) and thus is an index of the capillary supply to that fibre and considers the influence of contiguous capillary supply areas in terms of 'supply equivalents' at maximum perfusion/consumption. The number of domain overlapping a fibre (DAF) provides an index of any capillary supplying that fibre, and DAF and LCFR thus give complementary information. Using the assumption that 1 mol of oxygen is 22.4 L and the density of muscle is 1 $kg \cdot L^{-1}$, the MO_2max in a domain can be calculated in $pL \cdot min^{-1} \cdot mm^{-1}$ and volume specific MO_2max in $mL \cdot kg^{-1} \cdot min^{-1}$ (Bekedam et al., 2003).

Statistics

Stepwise regression was performed to assess the impact of fibre type, size and mass-specific MO_2max on LCFR and DAF. The correlations between MO_2max and domain area were

130 determined by Spearman correlation coefficients, as Shapiro-Wilk tests indicated that the data
131 were not normally distributed.

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134 **Results and Discussion**

135 We confirm that the local capillary to fibre ratio (LCFR) correlated positively with fibre size
136 (FCSA) in both human vastus lateralis (VL) (Fig. 2A; R=0.576, and R=0.625 when also
137 including mass-specific MO_2max in the model; both P<0.001) and soleus muscle (Fig. 2B;
138 R=0.578 and R=0.591, respectively; P<0.001). LCFR per fibre perimeter, a measure of the
139 capillary-fibre contact area, was positively related to the mass-specific MO_2max in both VL
140 (Fig. 2C; R=0.329; P<0.001) and soleus (Fig. 2D; R=0.138; P=0.002) muscles. Stepwise
141 linear regression revealed that the number of domains supplying a fibre (DAF) – a
142 functionally more realistic index than 'capillaries around a fibre'- was primarily determined
143 by FCSA; correlations improved when mass-specific MO_2max was also included in the
144 model (VL: R=0.470 *vs*. 0.508; both P<0.001; Soleus: R=0.497 *vs*. 0.510; both P<0.001).
145 Only in the soleus did inclusion of fibre type improve the correlation further (R=0.521;
146 P=0.043). Intriguingly, in both VL (Fig. 2E) and soleus (Fig. 2F) the MO_2max per capillary
147 varied from almost 0 to more than 1,000 $pL \cdot mm^{-1} \cdot min^{-1}$. Also, MO_2max was positively
148 correlated with domain area in VL (Fig. 2E) and soleus (Fig. 2F). Thus, capillaries with
149 larger oxygen supply areas supply a larger volume of mitochondria, and hence support a
150 potentially larger maximum oxygen flux. These observations may require a fundamental
151 review of ideas about determinants of local muscle capillary supply.

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153 Even at the whole muscle level, a poor correlation with gross capillarisation was found in
154 several species across a 17-fold range in muscle oxidative capacity (Maxwell et al., 1980).
155 Capillary growth can occur without an increase in oxidative capacity, e.g. following selective
156 stimulation of fast fibres (Egginton and Hudlicka, 2000). While there may still be temporal
157 coupling, as not all capillaries are perfused at any given moment, and perfused vessels may
158 have different flows adapted to the local demand for oxygen, it is unlikely that such
159 perturbations during exercise can account for the more than 1,000 $pL \cdot mm^{-1} \cdot min^{-1}$ range in
160 local MO_2max among capillaries, as even at rest all capillaries will have been perfused in as
161 little as 20 sec (Hargreaves et al., 1990). Another possibility is that the positive relationship
162 between local MO_2max and capillary domain size reflects a compensation for a reduced

oxygen diffusion gradient due to the decreasing microvascular PO_2 from the arteriolar to venular end of capillaries (Egginton and Gaffney, 2010). While these factors may help to match temporal oxygen supply and demand, they do not explain why local structural capillary supply correlates poorly with local maximal oxygen demand, a violation of the symmorphosis principle that assumes structures are matched to functional demand. While the heterogeneity of capillary spacing does have an impact on tissue oxygenation (Egginton and Gaffney, 2010), it is apparently not regulated to maintain MO_2max per capillary. The capillary-fibre exchange area, however, may be a better reflection of the capacity for oxygen flux, as suggested by the greater correlation between peak oxygen consumption with the capillary-fibre contact area than with capillary density (Hepple et al., 1997). Lack of significant correlations between fibre oxidative capacity and the local capillary density in both rat and human muscle (Wust et al., 2009), but significant correlations between LCFR or DAF per fibre perimeter and fibre oxidative capacity, support this suggestion. Finally, the high volume of mitochondria in larger domains may serve to enhance flux of oxygen by exerting an extraction pressure, hence increasing total respiration rate, even if individual mitochondria are working submaximally under conditions of reduced oxygen tension. It is striking, however, that the local capillary supply was more tightly related to FCSA than fibre oxidative capacity. Consistent with these observations, fibre hypertrophy and angiogenesis during muscle overload have a similar time course (Plyley et al., 1998), further supporting a coupling between fibre size and local muscle capillarisation. Such a coupling is in part explicable by the fact that not only muscle fibres and satellite cells, but also endothelial cells act as mechanotransducers and may both, in response to mechanical deformation, secrete factors with reciprocal effects (Christov et al., 2007).

Whatever the cause of this novel observation, the data indicate that 1) maximal oxygen demand supported by individual capillaries varies enormously (more than 1,000 $pL \cdot mm^{-1} \cdot min^{-1}$) and 2) muscle fibre size rather than local maximal oxygen demand is a prime determinant of local muscle capillarisation.

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Author contributions A.B. performed the experiments, analysis and data interpretation. S.E. and H.D. interpreted the data and wrote the manuscript. H.D. designed the experiments, helped with the analysis and data interpretation, and wrote the first draft of the manuscript. J.R. and B.G. took the muscle biopsies. All authors discussed the results and approved the final manuscript.

Author Information

The authors have no competing financial interests.

Correspondence and requests for materials should be addressed to H.D. (h.degens@mmu.ac.uk)

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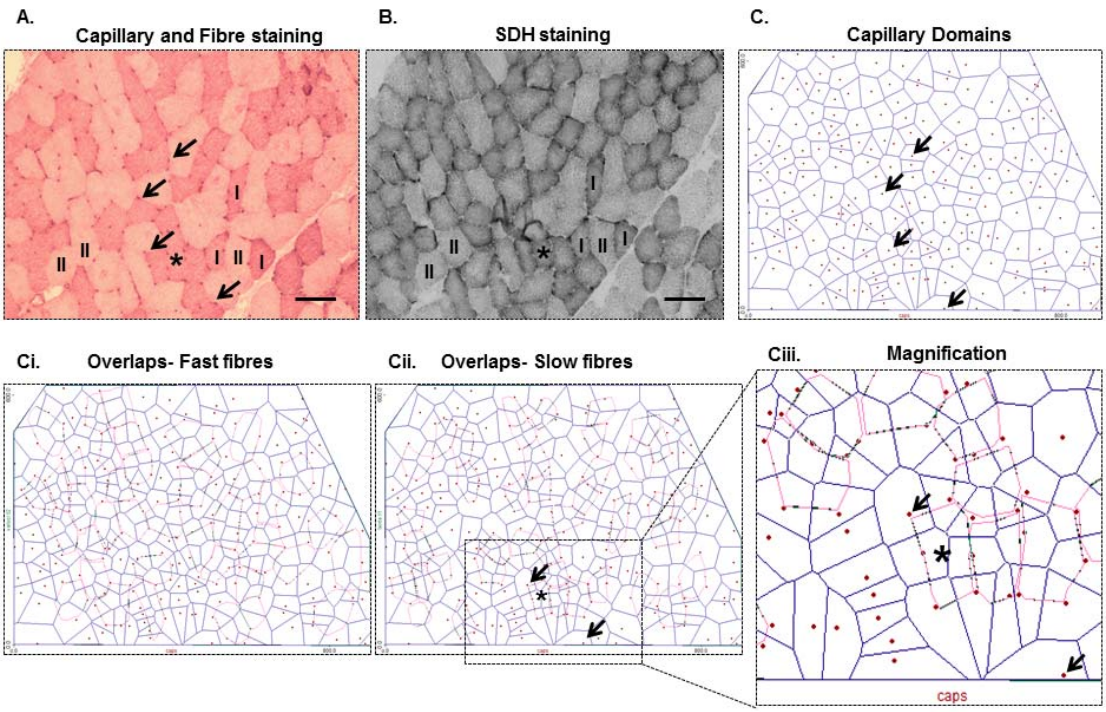
Figure legends

Figure 1. The relationship between muscle capillary supply area and aerobic capacity. Frozen sections of human vastus lateralis muscle biopsies stained with **A)** lectin (*Ulex europaeus*) to localise capillaries and **B)** succinate dehydrogenase as an index of mitochondrial activity. **C)** Capillary domains represent the area of tissue closer to a given capillary (red dots) than neighbouring capillaries. **Ci** and **Cii** show the overlap of domains with fast and slow fibres, respectively. **Ciii** shows a magnified region to illustrate overlap of domains (blue outlines) and fibres (pink outlines). I: Type I fibres; II: Type II fibres; arrows: corresponding capillary locations in each panel; * identifies the same fibre in each panel. Scale bar = 100 µm.

Figure 2. The relationship between local maximal oxygen demand (MO_2max) for individual capillaries and their respective domain area. There was a positive correlation between local capillary to fibre ratio (LCFR) and fibre cross-sectional area (FCSA) in **A**) human vastus lateralis (VL) (R=0.576, and R=0.625 when also including mass specific MO_2max in the model; both $P<0.001$) and **B**) soleus muscles (R=0.578 and R=0.591, respectively; $P<0.001$). The LCFR per fibre perimeter, a measure of the capillary-fibre contact area, was positively related to the estimated mass-specific MO_2max of fibres in **C**) the VL (R=0.329; $P<0.001$) and **D**) soleus (R=0.138; $P=0.002$) muscle. A positive correlation was also seen between estimated domain MO_2max and domain area in **E**) VL (R=0.750, $n=1443$ capillaries, $P<0.001$, R=0.822±0.017 for regression lines from each of the 10 individuals; mean±SEM) and **F**) soleus (R=0.697, $n=1742$, $P<0.001$, R=0.705±0.038) muscle. The VL and soleus contained 36±4% and 75±4% (mean±SEM) type I fibres, respectively.



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262 **Figure 1. The relationship between muscle capillary supply area and aerobic capacity.**

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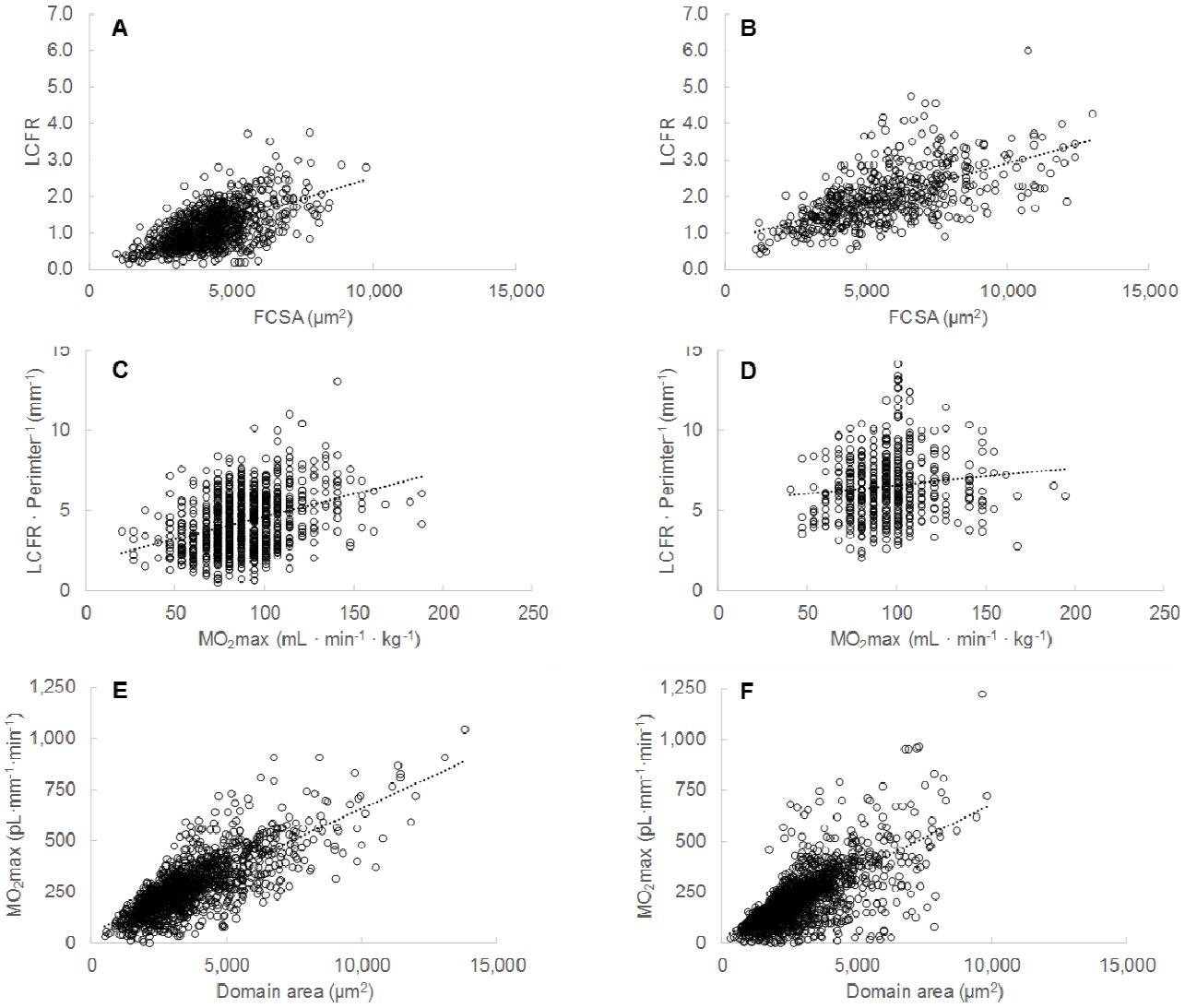

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265 **Figure 2. The relationship between the local muscle maximal oxygen demand (MO_2max)**
266 **from a capillary and domain area in human vastus lateralis (VL) and soleus muscles.**