Symposium Review

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Increased muscle blood supply and transendothelial nutrient and insulin transport induced by food intake and exercise: Effect of obesity and ageing

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Running title:

Transendothelial transport of nutrients and insulin to muscle fibres

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Abstract

This review concludes that a sedentary lifestyle, obesity and ageing impair the vasodilator response of the muscle microvasculature to insulin, exercise and VEGF-A and reduce microvascular density. Both impairments contribute to the development of insulin resistance, obesity and chronic age related diseases. A physically active lifestyle keeps both the vasodilator response and microvascular density high. Intravital microscopy has shown that microvascular units (MVUs) are the smallest functional elements to adjust blood flow in response to physiological signals and metabolic demand of muscle fibres. The luminal diameter of a common terminal arteriole (TA) controls blood flow through upto 20 capillaries belonging to a single MVU. Increases in plasma insulin and exercise/muscle contraction lead to recruitment of additional MVUs. Insulin also increases arteriolar vasomotion. Both mechanisms increase the endothelial surface area and therefore transendothelial transport of glucose, fatty acids (FA) and insulin by specific transporters, present in high concentrations in the capillary endothelium. Future studies should quantify transporter concentration differences between healthy and at risk populations as they may limit nutrient supply and oxidation in muscle and impair glucose and lipid homeostasis. An important recent discovery is that VEGF-B produced by skeletal muscle controls the expression of FA transporter proteins in the capillary endothelium and thus links endothelial FA uptake to the oxidative capacity of skeletal muscle, potentially preventing lipotoxic FA accumulation, the dominant cause of insulin resistance in muscle fibers.

Introduction

The aim of this symposium review is to provide an introduction and fill in the background knowledge for the subsequent reviews presented at Physiology 2014 in London in an Invited Symposium with the title: "Impact of physical activity, ageing, obesity and metabolic syndrome on muscle microvascular perfusion and endothelial metabolism". Increases in plasma insulin (following meal ingestion or insulin infusion) lead to modest increases in resting skeletal muscle blood flow and/or microvascular blood volume via an endothelial nitric oxide (NO) dependent mechanism. During aerobic endurance exercise the blood flow in the contracting skeletal muscles increases upto 80-fold compared to rest via a number of complementary mechanisms. In both cases more blood is present in or flowing through the muscle capillary bed, but the mechanisms by which these increases in muscle capillary blood flow occur are only partially understood. This review, therefore, will first explain the complex anatomy of the microvasculature in mammalian skeletal muscles, as a proper understanding of the anatomy is integral to understanding of the nature and mechanisms that control muscle capillary blood flow and the supply of oxygen, fuels and hormones to the skeletal muscle fibres. This will be followed by reflections on the metabolic rationale for the very large surface area of the endothelial cell layer (ECL) in the skeletal muscle microvasculature, especially the role that it plays in the transendothelial transport of oxygen, glucose, fatty acids (FA) and insulin from the lumen of the muscle capillaries into the interstitial fluid surrounding the skeletal muscle fibres. Finally, a summary will be given of the mechanisms by which meal-induced increases of plasma insulin and exercise induced increases in blood shear stress and in the interstitial concentration of vascular endothelial growth factor-A (VEGF-A) activate endothelial nitric oxide synthase (eNOS), dilate terminal arterioles (TAs) and thus increase capillary blood flow. The important role of these molecular mechanisms in maintaining optimal insulin sensitivity, glucose and lipid homeostasis, skeletal muscle perfusion during exercise and the angiogenic response to regular exercise will first be explained in healthy physically active individuals and intervened with a comprehensive summary of the most important impairments that occur in sedentary, obese and elderly individuals with and without chronic diseases.

Why are insulin induced increases in skeletal muscle blood perfusion important?

Research using contrast-enhanced ultrasound has shown that meal induced increases in the plasma insulin concentration lead to small (+50-80%) increases in the resting blood volume of the skeletal muscle microvascular bed in lean healthy individuals (Vincent *et al.* 2006). Convincing evidence has been delivered that this increase in micovascular blood volume leads to increased capillary recruitment in skeletal muscle both in rats (Rattigan *et al.* 1997) and humans (reviewed by Keske *et al.* unpublished symposium review). These increases are important, as they are part of the redistribution of the fuels that are absorbed with each of the main meals and, therefore, play an important role in the delivery of glucose, insulin and fatty acids to skeletal muscle as the main storage site. Following oral ingestion of a 92 gram glucose load leading to physiological increases in plasma insulin in healthy lean young males, 65-70% of the ingested glucose was taken up by peripheral tissues (Katz *et al.* 1983). Following oral ingestion of a mixed meal (40 g fat and 40 g carbohydrate) a substantial part of the meal-derived fat was taken up by cannulated forearm and abdominal subcutaneous adipose tissue as chylomicron-triglyceride (TG) and VLDL-TG (Bickerton *et al.* 2007). These studies collectively suggest that the peripheral tissues (primarily skeletal muscle and subcutaneous adipose tissue) make the largest contributions to the clearance of orally ingested glucose and lipid and, therefore, make an important contribution to postprandial glucose and lipid homeostasis. Postprandial increases in perfusion of the skeletal muscle microvascular bed, therefore, seem to be instrumental for glucose and lipid homeostasis and long-term metabolic health. However, studies with contrast enhanced ultrasound have shown that physiological, meal-induced and supraphysiological increases in plasma insulin achieved via a hyperinsulinemic euglycemic clamp fail to increase the blood volume that is present in the skeletal muscle microvascular bed in obese individuals (Clerk *et al.* 2006; Keske *et al.* 2009) and also in sedentary elderly individuals, type 2 diabetes patients and animal models of type 2 diabetes and insulin resistance (for references see reviews of Barrett *et al.* 2009 and Keske *et al.* unpublished symposium review). This suggest that the postprandial redistribution of the blood flow to skeletal muscle and potentially subcutaneous adipose tissue is reduced or absent in obese, elderly and insulin resistant individuals. This impairment explains the large transient increases that are seen in plasma glucose and TG concentrations following ingestion of mixed meals in these conditions. It is the prevalence and height of the elevated postprandial glucose excursions, which represents a high risk for development of type 2 diabetes (Abdul-Ghani & deFronzo, 2009) and which imposes a direct and independent risk in type 2 diabetes patients for the development of cardiovascular complications (Ceriello 2005). Increased postprandial plasma TG concentrations also presented an independent risk for the development of cardiovascular events and insulin resistance in a large cohort of healthy women (Bansal *et al.* 2007).

Why are exercise-induced increases in skeletal muscle blood perfusion important?

The microvasculature of human skeletal muscles has a complex 3D-structure and is subject to a large number of complementary blood flow regulation mechanisms (Mortensen & Saltin, 2014). This ensures that blood supply matches the metabolic demands of the muscle fibres under resting conditions and during exercise. The energy expenditure of skeletal muscle in comparison to other tissues and organs is relatively low at rest (0.5 watt/kg muscle), but can increase during aerobic exercise (e.g. during running at Olympic level marathon pace) to 80 watt/kg of contracting muscle. This is a 160-fold increase, which trained athletes are able to maintain for more than 2 h, because the density of both the mitochondrial network (ATP production capacity) and the microvascular network (oxygen and fuel supply) is much higher in endurance trained athletes than in sedentary individuals (Saltin *et al.* 1977; Saltin 1988). All the oxygen and blood borne fuels (glucose, fatty acids and lipoprotein TGs) required to meet this high energy demand are delivered to the muscle via the blood (Van Loon *et al.* 2001). It is for this reason that leg blood flow and perfusion of the muscle microvascular bed have to increase 10-80 fold (depending on running velocity and training status) during two-legged running. When the active muscle mass is small and the heart (cardiac output) is not a limitation, skeletal muscle blood flow in trained individuals can even increase 100-fold and reach perfusion rates of 300-400 ml/min per kg muscle (Andersen & Saltin, 1985; Richardson *et al.* 1993). A thorough knowledge of the anatomy of the muscle microvasculature to include the role played by the ECL and TAs in channeling blood borne fuels into the muscle interstitium is essential for a basic understanding of the mechanism by which aerobic endurance exercise leads to these 10 to100-fold increases in skeletal muscle microvascular perfusion and metabolic rate.

Functional anatomy of the microvasulature of skeletal muscle

Most of the historic thinking about the supply and utilization of oxygen and glucose in human skeletal muscle is based on spatial relationships of capillaries and skeletal muscle fibres measured in skeletal muscle cross-sections (e.g. Krogh 1918) (Fig. 1A). This remains common practice today as the generation of longitudinal and 3-dimensional (3D) images of the microvasculature in human muscle fibres currently is an insurmountable methodological challenge. The length of human muscle fibres is between 4 cm (soleus muscle) and 40 cm (sartorius muscle) (Wickiewicz *et al.* 1983; Ward *et al.* 2009), while the diameter of capillaries is in the 6-10 μm range. The capillaries, furthermore, are densely packed between the plasma membranes of neighbouring muscle fibres, which have a diameter of 50-90 μm depending on fibre type, age and training status. The imaging problems that result from this spatial organisation seem to be the reason that today there is very little published information on the 3D anatomical structure of the human skeletal muscle microvasculature. We, therefore, depend on information from other mammalian species to build up a basic understanding, keeping in mind that there may be differences with the human skeletal muscle.

Fig. 1B and 1C show images of a plaster cast of the highly branched network of the microvasculature of the mouse gluteus maximus muscle originally published by Segal (2005). A more recent educational description, linking anatomy to blood flow control in skeletal muscle, has been published by Segal & Bearden (2012) again in mouse gluteus maximus muscle. In brief upon leaving the heart, the convection of blood through large conduit arteries such as the brachial and femoral arteries rapidly conveys blood to skeletal muscle with minimal hemodynamic resistance. The large conduit arteries then split into muscular feed arteries, which are millimeters wide in humans and are still external to skeletal muscles. These feed arteries are positioned to control the total amount of blood entering the muscle and are assumed to present close to half of the total resistance to blood flow (Segal & Bearden, 2012). Upon entering skeletal muscle, feed arteries branch into the arteriolar networks, which are assumed to provide the major resistance to blood flow (Segal & Bearden, 2012). Primary or 1^{st} order arterioles branch into 2^{nd} and 3^{rd} order arterioles (Figure 2A). These intermediate branches distribute blood along the full length and full depth of the muscle and thereby control regional perfusion. Arising from the distributing 3^{rd} order arterioles are the 4^{th} order and terminal or 5^{th} order arterioles (TAs). The TAs control the perfusion of capillaries with red blood cells.

Capillaries are organised into microvascular units (MVUs)

Capillaries in mouse gluteus maximus muscle (Segal 1985; Segal and Bearden, 2012) and in hamster tibialis anterior muscle (Lund *et al.* 1987; Delashaw & Duling, 1988) are organised into microvascular units (MVUs). The latter represent the smallest volume of muscle to which blood flow and, therefore, oxygen, glucose and fatty acids (fuel) and insulin (hormone) delivery can be independently controlled. MVUs consist of all the capillaries arising from a common TA. TAs are oriented perpendicular to muscle fibres branching into a group of about 20 capillaries that run along the length of and between muscle fibres for a distance of about 1 mm. Usually blood flow in half of the capillaries is in one direction, and in the other half in the opposite direction, along the length of the same muscle fibres. The two sections of the MVU with flow in opposite direction have been named a "unit pair" (Lund *et al.* 1987). The capillaries empty on both sides into collecting venules (CV's, Fig. 2B). The volume of muscle tissue within an MVU with 2 unit pairs has been estimated at 0.2 mm^3 (or 2 mg), with average dimensions being 2 mm long, 0.5 mm wide, and 0.2 mm thick. This volume contains segments of about 20 different muscle fibres, which must originate from different motor units as the muscle fibres in a motor unit are spread over a much larger cross-sectional area.

Control of blood flow in microvascular units

In order to investigate the control of the blood flow in the capillaries of individual MVUs and potential differences between adjacent MVUs Lund *et al.* (1987) and Delashaw & Duling (1988) performed an impressive series of experiments in which they removed the skin and fascia from the tibialis anterior muscle in anesthetised hamsters. This allowed these researchers to use intravital epifluorescence microscopy to visualise the flow of red blood cells from the TA through capillaries to the CV in the 160-180 μm layer (one MVU deep) immediately below the surface of the muscle. When this preparation in control experiments was superfused with a Krebs-Ringer buffer that did not contain oxygen basically all the capillaries in all MVUs were continuously perfused with blood. Exposure to a superfusate containing 10% O_2 typically resulted in closure of approximately 50% of the capillaries, with the blood flow being simultaneously arrested in all capillaries of a given MVU by complete closure of the TA. An important observation in these studies was that in the MVUs, that remained to be perfused, the blood flow in all capillaries showed rhythmic oscillations, which were attributable to a spontaneous rhythmic change of the diameter of its TA. This phenomenon was called arteriolar 'vasomotion'. During vasomotion, flow in all of the capillaries of a given MVU tended to vary in synchrony with same phase and frequency (3-6 cycles per minute), while phase and frequency tended to vary between adjacent MVUs.

Delashaw & Duling (1988) in the same muscle preparation investigated the effects of 1) direct electrical stimulation leading to muscle contractions, 2) topical application of adenosine (vasodilator) and 3) topical application of phenylephrine (vasoconstrictor). The predominant response to these stimuli again consisted of a coordinated change in the blood flow in virtually all the capillaries of the investigated MVUs. Electrical stimulation of muscles in which blood flow was arrested in 95% of the MVUs led to the simultaneous recruitment of virtually all capillaries (of both unit pairs) in previously closed MVUs. Topical application of adenosine had a similar result, while topical application of phenylephrine resulted in the simultaneous arrest of the blood flow in all capillaries of previously perfused MVUs.

The study of Lund *et al.* (1987) also revealed that capillaries of neighbouring MVUs often run in parallel and overlap and cross with each other, implying that multiple MVUs supply oxygen, fuel and insulin to the same sections of the same muscle fibres. The flow in adjacent MVUs was both concurrent and countercurrent with 25-50% of the adjacent capillaries having a countercurrent flow. Suggestions have been made that the % countercurrent flow is around 50% for capillaries in deeper muscle layers originating from adjacent MVUs on both sides. Such a mixed concurrent-countercurrent flow pattern allows for the diffusion of oxygen between adjacent capillaries and on theoretical grounds has been suggested to give the most homogeneous type of tissue oxygenation (Grunewald & Sowa, 1977). In more recent studies (reviewed by Pittman (2013)) convincing evidence has also been presented that muscle arterioles also exchange oxygen via diffusion to adjacent capillaries (which they often cross) and that oxygen diffusion occurs between arteriole-venule pairs which often run next to each other over distances upto several mm (Fig 1B).

Relevance of these findings for resting and contracting human skeletal muscles

Although the above studies have generated a wealth of information on MVUs in skeletal muscle of anesthetised hamsters a major problem that remains is how to relate these findings to intact undisturbed human skeletal muscles in the *in vivo* resting state and during exercise or contraction. As the same structural organisation and similar size of MVUs has also been observed in rabbit tenuissimus muscle (Lindbom 1983) and in monkey skeletal muscle (Weibel, 1984), it is assumed in this review that this will also be the case for other mammalian species to include man. A limitation of the superfused hamster muscle preparations indicated by Lund *et al.* (1987) is that the PO_2 of the superfusion buffer has a major impact on vasoconstiction and vasomotion of TAs and therefore on capillary blood flow in MVUs. As such it was not possible for Lund *et al.* (1987) to decide what the 'normal' state in this muscle is. Is it the continuous perfusion of all MVUs and capillaries observed with a superfusion solution containing no oxygen (condition 1), or the mixture of closed MVUs and MVUs showing cyclic vasomotion observed with a superfusion solution gassed with 10% oxygen (condition 2)? The vasodilatation observed during chronic electrical stimulation and in response to adenosine and the vasoconstriction seen in response to phenylephrine in condition 2 (Delashaw & Duling, 1988) is very similar to *in vivo* effects of voluntary exercise and of arterial infusion of adenosine and phenylephrine on limb and local muscle blood flow seen in humans *in vivo*. Cyclic vasomotion of the skeletal muscle microvasculature has also been studied in rats *in vivo* with laser Doppler flowmetry (LDF) with insertion of the probe in the tibialis muscle parallel to the muscle fibre direction (Newman *et al.* 2009). This study revealed that vasomotion occurred with a frequency in the range of 6 cycles per minute, which is in the same frequency range as observed by Lund *et al.* (1987) in MVUs of the hamster tibialis anterior muscle using a superfusion solution gassed with 10% oxygen.

Assumptions on the regulation of capillary blood flow in human skeletal muscle currently not supported by human data

In the remainder of this review, therefore, the assumptions will be made that there are similar size MVUs in human skeletal muscles fibres as in other mammalian species and that part of these MVUs will be closed in the normal resting state, while others undergo cyclic vasomotion with a large variation in the perfusion rate between adjacent MVUs. Cyclic vasomotion of TAs in combination with a mixed concurrent-countercurrent flow pattern in adjacent capillaries (originating from adjacent MVUs) is assumed to play an important role in ensuring that skeletal muscles are evenly perfused by periodically redistributing blood from one MVU to adjacent ones. Vasomotion will allow switches in time both in recruitment of different MVUs and in the overall blood perfusion rate of a given MVU by step-wise changes in the lumen of the common TA. Vasomotion as such may contribute to the local blood flow control mechanism that can lead to an even perfusion rate over the full length of the upto 40 cm long multinucleated muscle fibres in human skeletal muscle and may also help to ensure that the approximately 20 fibre sections that are present in a single MVU are perfused in proportion to their metabolic demand. Please note that future research is required to confirm these assumptions.

Anatomy and function of the endothelial cell layer in the microvasculature of human skeletal muscle

Every blood vessel in the human vasculature is covered on the luminal side with a continuous monolayer of ECs (ECL). The total surface area of the ECL in an adult human has been estimated to cover > 700 m^2 and to have a weight of about 700 g (Wolinsky, 1980). This makes the ECL into one of the largest diffuse tissues in the human body with a significant weight despite the 0.3 μm thickness. The ECL of all arteries and veins in our body together covers only about 6 m^2 (Fig. 1D), while the ECL of the microvasculature (arterioles, capillaries and venules) contributes 99% of the total surface area. Of the latter 85% (about 600 m^2) is present in capillaries (Fig. 1D). As skeletal muscle in a 70 kg lean physically active adult has a mass between 35 kg and 40 kg and has a higher capillary density than other tissues (with exception of the heart), estimates are that at least 400 m^2 of the ECL is present in the skeletal muscle microvasculature, again with the largest surface area in the dense network of capillaries (Wolinsky, 1980). The metabolic rationale behind this distribution seems to be that the ECL in muscle capillaries contains all the enzymes that facilitate the transendothelial transport of glucose, amino acids, fatty acids and insulin into the interstitial fluid that surrounds the muscle fibres and that transport capacity, therefore, can be increased by recruiting a larger endothelial surface area at times of increased metabolic demand (eg following meal ingestion and during exercise).

Transendothelial transport of oxygen, nutrients and insulin

ECs in the skeletal muscle microvasculature form a continuous mono-layer and as such present the first potential barrier for the transport of oxygen, nutrients and insulin from the vascular compartment to the interstitial fluid surrounding the skeletal muscle fibers. The number of studies that have evaluated the impact of this endothelial barrier on the supply of oxygen and nutrients to the interstitium of skeletal muscle is surprisingly small. As far as the authors are aware it is not known today which % of the oxygen released from hemoglobin and the red blood cells is diffusing into the interstitium of skeletal muscle via the paracellular route (through the tight junctions between neighbouring ECs) and via the transcellular route (transendothelial transport (TET) from the luminal to the abluminal membrane through the cytosol of ECs). Still to answer the question whether O_2 diffusion from capillary red bloods cells into the muscle interstitium limits VO_{2max} in healthy humans and in sedentary obese and elderly individuals with and without type 2 diabetes and/or cardiovascular disease and ischemia, this is important information (Roca *et al.* 1989; Spires *et al.* 2012).

The review of Mann *et al.* (2003) comes to the conclusion that glucose transport across the luminal and abluminal membrane of the endothelium of the blood brain barrier occurs via a facilitative, energy-independent and saturable transport process by the GLUT-1 transporter. GLUT-1 has also been detected in the endothelium of the blood-retinal barrier, the cornea, and of freshly isolated and cultured bovine aorta ECs (bAECs). Total glucose transport rates across the retinal endothelium exceeded the metabolic rates of the retinal endothelium by far suggesting that GLUT-1 in ECs facilitates transendothelial transport (TET) of glucose into the underlying tissues. The driving force for glucose transport by GLUT-1 is the concentration gradient between the capillary lumen and the interstitium. Davey *et al.* (2007) using immunogold EM methods have shown that in rat heart GLUT-1 is the only glucose transporter present in the capillary ECs, while GLUT-4 was the dominant glucose transporter in cardiomyocytes. Bradley and Wagenmakers (unpublished data) using immuno-staining microscopy observed an intense GLUT-1 signal in ECs of capillaries in human vastus lateralis muscle and no GLUT-4, while the reverse was seen in the muscle fibres (Bradley *et al.* 2014), implying that the same GLUT distribution is present in human skeletal muscle as in rat heart. These data suggest that the main transport route of glucose is through the transcellular route with glucose transport facilitated by GLUT-1.

In addition Mann *et al.* (2003) in their review come to the conclusion that ECs lining the blood-brain barrier and blood retinal barrier and cultured bAECs all express a large number of specific amino acid transport systems for practically all amino acids. There is no information on the presence of these amino acid transporters in the capillary endothelium of skeletal muscle, but the assumption is made that amino acids again are primarily transported into the skeletal muscle interstitium via the transcellular route.

Fatty acids (FA), chylomicron triglycerides (CM-TG) and very low density lipoprotein triglycerides (VLDL-TG) are important fuels for human skeletal muscle both at rest (Bickerton *et al.* 2007) and during exercise with peak oxidation rates occurring at intensities of 55-65% VO_{2max} (Van Loon *et al.* 2001). Plasma FA and TG are again delivered to muscle fibers via the capillaries. The enzyme lipoprotein lipase (LPL) is attached to the luminal membrane of capillary ECs and is responsible for the breakdown of CM-TG and VLDL-TG to FA. Until recently it was an open question how these FA are subsequently transported into the skeletal muscle interstitium for subsequent uptake in skeletal muscle, although the first study that observed a high concentration of FAT (fatty acid translocase)/CD36 in the endothelium of skeletal muscle, heart and adipose tissue of mice dates back to 1995 (Greenwalt *et al.* 1995). This observation was confirmed in 2004 with high quality confocal immuno-fluorescence microscopy images clearly showing that the FAT content of the capillary endothelium is much higher than in the skeletal muscle fibres (Vistisen *et al.* 2004). FAT/CD36 today is regarded to be the most important FA transporter responsible for FA entry into skeletal muscle (Glatz *et al.* 2010; McFarlan *et al.* 2012). This conclusion is based on experiments with giant plasma membrane vesicles suggesting that postprandial increases in insulin and exercise/contraction lead to translocation of FAT from hypothetical microsomal storage depots (similar to GLUT-4 storage and translocation) to the plasma membrane of skeletal muscle fibers (reviewed by Glatz *et al.* 2010). However, Vistisen *et al.* (2004) using confocal immunofluorescence microscopy only detected FAT/CD 36 in the plasma membrane of the skeletal muscle membrane also in resting biopsies obtained in the fasted state in an intensity that was only a fraction of the stain in the capillary ECs. Hagberg *et al.* (2010) also using immuno-staining methods observed that FA transporter proteins (FATP3 and FATP4) were expressed abundantly in the ECs of capillaries of mouse skeletal muscle, heart and adipose tissue. These proteins also facilitate the transport of FAs across the luminal and abluminal membrane. Iso *et al.* (2013) observed that FA binding proteins (FABP4 and FABP5) are abundantly expressed in the ECL of mouse skeletal muscle

capillaries and venules (but not in arterioles, arteries and aorta). These proteins function as the transport vehicle for FAs diffusing through the cytosol from the luminal to the abluminal membrane of ECs. In skeletal muscle of FAT/CD36 KO mice (McFarlan *et al.* 2012), with a low expression of FATP3 and FATP4 (Hagberg *et al.* 2010), and with a double knock out for FABP4 and FABP5 (Iso *et al.* 2013) skeletal muscle uptake of FA was substantially reduced, while the uptake of glucose and GLUT-4 expression in skeletal muscle was remarkably increased. Collectively these data provide convincing evidence that the main route for transport of FA across the ECL is via the transcellular route, that a low protein expression of FAT/CD36, FATPs and FABPs limits FA uptake in mouse skeletal muscle and that capillary ECs act as the gate-keepers for uptake of FAs into skeletal and as such may play a very important role in the distribution of lipids between skeletal muscle, subcutaneous adipose tissue and ectopic fat stores and, therefore, the mechanisms leading to insulin resistance of the skeletal muscle fibres (Fig. 3).

Transport of insulin from the capillary lumen into the skeletal muscle interstitium has been investigated in great detail in a series of elegant studies by the research group of Professor Eugene Barrett primarily in cultured bovine aortic ECs (bAECs) (Wang *et al.* 2008, 2009, 2011, 2012, 2013) and in rat skeletal muscle *in vivo* (Wang *et al.* 2006). These studies are also placed in the context of the related human literature in reviews of Barrett *et al.* (2009 and 2011). Both in the *in vivo* study investigating the rectus muscle of the rat and in *in vitro* studies with bAECs ECs rapidly took up fluorescein isothiocyanate- (FITC-) labelled insulin. The fluorescent label allowed quantification of insulin uptake with confocal fluorescence microscopy. In the *in vivo* study in rats (Wang *et al.* 2006) the insulin concentration in ECs by far exceeded the concentration in plasma and muscle interstitium. Both the insulin receptor and IGF-1 receptor mediated insulin transit through monolayers of bAECs in a process involving the caveolae, which are present in the lipid rafts in the plasma membrane (PM) (Wang *et al.* 2006). Adding insulin provoked the prompt translocation of both caveolin-1 and eNOS to the PM and led to co-immunoprecipitation of these proteins, suggesting that insulin signaling brings these proteins together in lipid rafts in the PM (Wang *et al.* 2009). Insulin uptake required intact insulin signaling via both the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling cascades (Wang *et al.* 2008). Pre-incubation of bAECs with the pro-inflammatory cytokines TNF-α and IL-6 significantly diminished insulin uptake, at least in part by inhibiting caveolin-1 expression (Wang *et al.* 2011). Wang *et al.* (2012) found that exposure of bAECs to insulin within 5 min induced

substantial cortical actin filament remodeling. The remodeling was inhibited by inhibition of PI3K or MAPK signaling and disruption of actin microfilaments and lipid rafts with specific inhibitors. Knockdown of either caveolin-1 or Akt led to complete elimination of the insulin-induced cortical actin filament remodeling. The conclusion of Wang *et al.* (2012) was that insulin-induced cortical actin filament remodeling is required for TET of insulin and depends both on intact PI3K/Akt signaling and the presence of caveolin-1 in intact lipid rafts. Wang *et al.* (2013) observed that L-NAME (NOS inhibitor) pretreatment of bAECs blocked FITC-labelled insulin uptake, whereas pretreatment with L-arginine (converted into NO by eNOS) and the NO-donor sodium nitroprusside (SNP) enhanced insulin uptake. SNP also reversed the inhibition of insulin uptake in bAECs by L-NAME, wortmannin (PI3K inhibitor) and TNFα. SNP increased TET of ^{125}I-labeled insulin with 40% in bAEC monolayers. SNP finally increased S-nitrosylation of the protein tyrosine phosphatase PTP1B which dephosphorylates tyrosine residues on the IR and IRS-1 and IRS-2. S-nitrosylation led to inactivation of PTP1B activity and an increase in insulin signaling leading to Ser^{473} phosphoryalation of Akt. The conclusion of Wang *et al.* (2013) was that a high endothelial NO concentration directly promotes insulin transport into ECs and TET of insulin via a mechanism that involves enhancement of S-nitrosylation, inhibition of the activity of PTP1B and enhanced insulin signaling. As enhanced insulin signaling activates eNOS (Fig. 2) and increases endothelial NO production, this implies that insulin stimulates its own uptake in ECs lining skeletal muscle capillaries (Barrett *et al.* 2011).

Permeability surface area product as measure of transendothelial glucose transport

Gudsbjörnsdóttir *et al.* (2003) have combined forearm arteriovenous cannulations in healthy lean men with microdialysis of the brachioradialis muscle to measure arterial, venous and interstitial glucose concentrations. Using this approach the authors were able to make a direct calculation of the permeability surface area product (PS) of the ECL for glucose in this muscle. The PS depends both on the surface area of the ECL that is available for glucose uptake (and therefore on number of simultaneously perfused MVUs) and the permeability of the ECL for glucose. As GLUT1 is the only transporter detected in the capillary endothelium of human skeletal muscle (Bradley & Wagenmakers, unpublished), it is the GLUT1 protein content that determines permeability. A traditional oral glucose tolerance test increased the PS for glucose and the glucose uptake by the muscle about 2-fold in the 90-150 min period after glucose ingestion. During a steady state euglycemic hyperinsulinemic clamp PS for

glucose increased 10-fold and glucose uptake by the muscle increased 8-9 fold. These data provide convincing evidence that oral ingestion of glucose and insulin infusion lead to substantial increases in the recruitment of endothelial surface area in the muscle microvasculature and that these lead to proportional increases in muscle glucose uptake. As glucose transport across the ECL is mediated by the insulin insensitive GLUT-1 transporter it is unlikely that increases in endothelial permeability make a major contribution to the observed increases in PS, although increases by other currently unknown activation mechanisms cannot be excluded.

Gudsbjörnsdóttir *et al.* (2005) measured the PS for glucose and insulin during a steady state euglycemic hyperinsulinemic clamp in male individuals with type 2 diabetes (T2D) and age- and weight-matched obese controls with normal fasting glucose. The PS for glucose was 2.2-fold lower in the patients with T2D diabetes than the obese controls and muscle glucose uptake was 1.7-fold lower in the patients. The absolute value for the PS for glucose in the lean men during the steady state phase of the euglycemic hyperinsulinemic clamp was 2.3 ± 0.9 ml/min.100g in the study of Gudsbjörnsdóttir *et al.* (2003) and the equivalent values in the obese controls and type 2 diabetes patients in Gudsbjörnsdóttir *et al.* (2005) were 1.1 ± 0.2 and 0.5 ± 0.1 ml/min.100g. The corresponding muscle glucose uptake rates were 4.4 ± 1.2, 3.0 ± 0.4 and 1.8 ± 0.3 μmol/min.100g in the lean healthy men, obese controls and type 2 diabetes patients, respectively. These data confirm that the patients with type 2 diabetes have by far the lowest insulin stimulated capacity for transcapillary passage of glucose into the muscle interstitium and that obese men have a lower capacity than lean men. The differences are likely to at least in part be the result of the reduction in the surface area resulting from reduced insulin stimulated increases in microvascular dilation and blood volume (Keske *et al.* 2009), but differences in endothelial GLUT-1 content between these groups can also make a contribution.

Spires *et al.* (2012) have used experimental measurements in combination with a computational model to calculate the endothelial PS during incremental moderate intensity exercise and among others observed a gradual increase in PS, which showed a linear relationship with the increase in blood flow through the skeletal muscle capillary bed. How can we translate this into recruitment of MVUs and capillaries in skeletal muscle? For reasons explained in the previous sections it is not known today which % of the MVUs in human (or animal) skeletal muscle is perfused in the resting state at a given time. The

occurrence of vasomotion with large differences in phase and frequency between different MVUs also implies that the MVUs are constantly switching between the closed and open position in resting muscles. However, given the fact that oxygen consumption by the combined skeletal muscles and blood flow through the muscle capillary bed increases 80 to 100-fold going from rest to maximal exercise (Andersen & Saltin, 1985) it seems reasonable to assume that the % of MVUs in the open position is close to 100% during maximal exercise and much lower (maybe as low as 5-10%) in the resting state. However, over the course of minutes it is likely that all MVUs have been in the open position part of the time. This then implies that only a fraction of the ECL surface area will be available for transport of oxygen, glucose and FAs in the resting state, while most of this surface area will be recruited during maximal exercise. A gradual increase in the number of MVUs that are constantly open would provide a powerful mechanism to ensure that the supply of blood borne fuels and oxygen meets the growing energy-demand of the contracting muscle fibres. The authors of this review want to make the reader aware here that Poole *et al.* (2013) have proposed that also at rest all capillaries in skeletal muscle are simultaneously perfused, but only over a fraction of their length with longitudinal recruitment occurring during exercise. It, however, is not clear to the authors of this review how such a recruitment pattern can be made compatible with the blood flow patterns to include vasomotion that has been observed in MVUs among others by Lund *et al.* 1987 and Delashaw & Duling (1988).

Shear stress signal function of the endothelial glycocalyx

The luminal surface of the endothelium is covered with a brush like glycocalyx layer (glycoproteins and proteoglycans), which is approximately 0.5 μm thick in capillaries and 4.5 μm in arteries (Reitsma et al 2007; Weinbaum et al 2007). An important function of the glycocalyx is the translation of hemodynamic forces (shear forces exerted by flowing blood and individual blood cells passing through the narrow lumen of a TA) into vasodilatory responses during exercise. As such mechanical forces exerted on the glycocalyx are likely to be early signalling events leading to eNOS activation and exercise training induced increases in skeletal muscle microvascular density.

The molecular mechanisms by which insulin increases muscle capillary blood flow

There is today compelling evidence both in rat and humans that an insulin-mediated increase in microvascular perfusion of skeletal muscle is a conditional early event leading to increases of the transendothelial transport (TET) of both glucose and insulin into the interstitial fluid that surrounds the muscle fibres (reviewed by Barrett et al. 2009 & 2011 and Keske *et al.* unpublished symposium review). Once the insulin concentration in the interstitium starts to increase (15-20 min after ingestion of a glucose load or start of an euglycemic hyperinsulinemic clamp, this leads to subsequent activation of the insulin signalling cascade,

GLUT-4 translocation to the plasma membrane and increased glucose uptake in skeletal muscle fibres (Vincent *et al.* 2004).

Important early information on the molecular mechanisms by which insulin is able to recruit additional blood flow in muscle capillaries has come from studies in cultured ECs (for references see Vincent *et al.* 2003; Wagenmakers *et al.* 2006; Muniyappa *et al.* 2007; Keske *et al.* unpublished symposium review). These studies identified an insulin-signalling cascade (Insulin receptor/IRS-1/PI3-Kinase/PDK1/Akt/eNOS; Fig. 2), activation of which leads to increased production of nitric oxide (NO). NO produced *in vivo* by the ECL is a potent vasodilator acting upon the smooth muscle cell layer in skeletal muscle arterioles. Insulin was found to activate the enzyme endothelial nitric oxide synthase (eNOS) among others by means of ser^{1176} phosphorylation in cultured rat ECs and ser^{1177} phosphorylation in cultured human ECs. Other signals found to activate eNOS in cultured ECs via $ser^{1176/1177}$ phosphorylation are fluid shear forces exerted on a cultured EC monolayer and exposure of cultured ECs to vascular endothelial growth factor-A (VEGF-A).

With the current understanding of the role played by TAs in the blood supply to MVUs, the likely eNOS activation site by which physiological increases in insulin following meal ingestion lead to the simultaneous recruitment of a larger number of MVUs are the TAs. This has been confirmed by the observation of Cocks *et al.* (2013b) that 80 min after the start of a hyperinsulinemic euglycemic clamp in lean Zucker rats eNOS ser^{1176} phosphorylation was increased by 14% ($P< 0.05$) in the ECL of TAs, while no increase was seen in the capillaries.

Newman *et al.* (2009), using Laser Doppler Flowmetry of skeletal muscle, observed that a hyperinsulinemic euglycemic clamp in rats induced an increase in the relative amplitude of the myogenic component of vasomotion which coincided with an increase in hindlimb glucose uptake. This observation suggested that the ability of insulin to recruit muscle microvascular blood flow is at least in part the result of increased arteriolar vasomotion in skeletal muscle. Vasomotion of TAs is also the likely mechanism to expose the ECL of a previously closed TA to the higher insulin concentration of the incoming blood. It is also possible that TET of insulin in open MVUs leads to increases in the interstitial insulin concentration and thus to the activation of the insulin receptor or IGF-1 receptor in the abluminal membrane of closed MVUs. There currently is no information on the density of these receptors on the luminal and abluminal endothelial membrane, implying that this back-door mechanism cannot be excluded. It also cannot be excluded that adenosine or ATP or other vasodilators play a role in giving luminal plasma insulin access to the luminal membrane of the ECL of closed TAs in the period that the plasma and interstitial insulin concentrations are rising. The adenosine may be produced by ECs, vascular smooth muscle cells and also skeletal muscle fibres in which the metabolic rate is increased by rises in interstitial insulin that are not matched by the oxygen supply as most of the MVUs serving

that muscle fibre are in the closed position. Collectively these mechanisms will lead to an increase in the available capillary surface area and, therefore, result in increases in transport rates of insulin and glucose without the necessity of a measurable increase in total muscle blood. The latter has been observed in several studies (e.g. Raitakari *et al.* 1996). Insulin as mentioned before also increases its own TET via a mechanism that involves eNOS activation, enhancement of S-nitrosylation of PTP1B inhibiting its phosphatase activity and therefore enhancing endothelial insulin signaling (Fig. 8 in Wang *et al.* 2013).

There also is evidence that there are impairments in the insulin-induced activation of eNOS in animal models of insulin resistance and patients with type 2 diabetes. Cocks *et al.* (unpublished) did not find an increase in eNOS ser^{1176} phosphorylation during an euglycemic clamp in the ECL of TAs in obese Zucker rats, while a significant 14% increase was seen in lean Zucker rats. Tabit *et al.* (2013) reported an insulin induced increase in eNOS^{1177} in freshly isolated venous ECs from nondiabetic controls, but not in diabetic patients (P=0.003), consistent with endothelial insulin resistance in type 2 diabetes. This study also provided convincing evidence that oxidative stress (nitrotyosine levels) and inflammation (nuclear factor κB activation) was higher in ECs from the patients and that protein kinase C-β activity was implicated in the endothelial insulin resistance. These data suggest that the mechanisms leading to insulin resistance in the ECL and in muscle fibres are quite similar (for comparison see Fig. 3; for further evidence and references see Wagenmakers *et al.* 2006 and Muniyappa *et al.* 2007).

The molecular mechanisms by which an exercise-induced increase in blood shear stress increases muscle capillary blood flow

Endurance exercise at moderate intensities always leads to parallel increases in capillary perfusion and total muscle perfusion. Endurance exercise for 1 h at 65% VO_{2max} led to eNOS activation by means of increased eNOS ser^{1177} phosphorylation in a mixture of muscle capillaries and TAs (Cocks *et al.* 2012; Cocks *et al.* 2013a). The increase in shear stress sensed by the endothelial glycocalyx is likely to play a role in the activation mechanism. This again will allow recruitment of more MVUs and a higher blood flow rate in the recruited MVUs by maintaining a high blood flow rate for a longer fraction of the time via increased vasomotion activity. In addition, despite lack of published data to confirm this, such increases in eNOS phosphorylation are also likely to occur in lower order arterioles and the muscular feed arteries. This assumption is based on the observations of van Teeffelen & Segal (2000) that as metabolic demand of the contracting muscle fibres increases, vasodilatation ascends progressively from the TAs into larger arterioles and their feed arteries. The induction of this mechanism is NO independent (Budel *et al.* 2003). Research of Segal & Jacobs (2001) generated evidence that electrical signals generated by ECs can travel rapidly along the ECL from TAs into their parent blood vessels, thereby leading to vasodilatation of the larger arterioles, resistance and feed arteries. This mechanism is called conductive vasodilatation (Segal & Jacobs, 2001) and contributes to the increases in skeletal muscle perfusion during exercise. The resultant increase in shear stress in these vessels is likely to lead to eNOS activation in lower order arterioles and muscular feed arteries. Acute endurance exercise has also been shown to activate eNOS via ser^{1177} phosphorylation in mouse aorta (Zhang *et al.* 2009; Cacicedo *et al.* 2011).

The molecular mechanisms by which an exercise-induced increase in the interstitial VEGF-A concentration increases muscle capillary blood flow

Vascular endothelial growth factor-A (VEGF-A) is regarded to be the central angiogenic factor in skeletal muscle capillary growth in response to exercise training interventions (Hoier & Hellsten, 2014). Skeletal muscle fibres are an important source of VEGF-A in humans. Muscle fibres contain substantial VEGF-A stores in small vesicles present between the myofibrils, with smaller amounts of VEGF-A being present in cells located in the interstitial fluid that fills the space between capillaries and muscle fibres, to include ECs and pericytes. Hoier and Hellsten (2014) in their recent review propose that VEGF-A-containing vesicles translocate to the plasma membrane and secrete their content into the interstitial fluid during exercise. As VEGF-A mRNA expression is primarily increased after exercise, it is also proposed that the VEGF-A stores lost through secretion during exercise are rapidly replenished in the 12-24 h period after exercise. As VEGF-A is secreted during exercise it is impossible to separate the contribution made by increased blood shear stress and by VEGF-A to the increase in eNOS ser^{1177} phosphorylation seen by Cocks *et al.* (2012 & 2013a) in the ECL of a mixed population of TAs and capillaries after 1 h of cycling exercise at 65% VO_{2max}. As VEGF-A is approaching the ECL via the skeletal muscle interstitium it may also help to activate eNOS via receptors on the abluminal membrane of TAs to thus recruit previously closed MVUs.

Activation of eNOS by increases in blood shear stress and interstitial VEGF during aerobic endurance exercise, therefore, are likely to both contribute to the mechanisms that lead to the recruitment of additional MVUs and maintenance of a higher mean blood flow in the recruited MVUs, but it is clear that there is an interaction between several locally produced vasodilators (NO, prostaglandins, ATP, adenosine) (for recent review see Mortensen & Saltin, 2014). There is evidence that NO and prostaglandins are the main vasodilators with a compensatory formation of the other vasodilator occurring when one is inhibited (Mortensen & Saltin, 2014).

VEGF-B controls the expression of endothelial FA transporter proteins

Four closely related vascular endothelium growth factor (VEGF) isoforms are expressed in human skeletal muscle (VEGF A-D). VEGF-A is best characterised because of its role in the angiogenic effect of exercise training. Until recently the role of VEGF-B in regulating blood vessel function was not known. Hagberg *et al.* (2010) made the observation that VEGF-B in mouse skeletal muscle co-expressed with a large cluster of nuclear genes encoding for mitochondrial proteins. This co-expression was unique for VEGF-B. Using a broad spectrum of molecular research methods, freshly isolated and cultured ECs and transgenic animal models, this study generated convincing evidence for the existence of a novel mechanism, which couples uptake and transendothelial transport of FA to the capacity of mitochondrial β-oxidation (Fig. 4). In brief VEGF-B produced by skeletal muscle fibres is released to the interstitium and diffuses to the abluminal membrane of ECs in the capillary ECL. Here, it binds to VEGF receptor 1 and neuropilin 1 which are both expressed by the ECL. Signaling of VEGF-B via a PI3K dependent mechanism then leads to the abundant expression of FATP3 and FATP4 in the ECL and incorporation into the luminal and abluminal membrane and, therefore, leads to increased transendothelial transport of FAs into the muscle interstitium where they diffuse to the muscle plasma membrane. Hagberg *et al.* (2010) also performed a study in which ^{14}C-oleate (a long-chain FA) was given to wildtype and VEGF-B (-/-) mice by oral gavage and the incorporation of the radioactive long-chain FA was measured in tissues. The incorporation into muscle, heart and brown adipose tissue of VEGF-B (-/-) mice was much lower than in tissues of wild-type mice. In the VEGF-B (-/-) mice most of the radioactive FA accumulated in white adipose tissue pads. VEGF-B (-/-) mice also gained more weight with age and developed obesity as evidenced by 60-90% larger fat pads and a higher body fat mass.

Conclusions

The most important conclusion of this review is that the monolayer of ECs lining the lumen of skeletal muscle capillaries contains high concentrations of binding and transporters proteins, which help to facilitate the transport of glucose (GLUT1), FAs (FAT/CD36, FATP4, FATP5), insulin and amino acids across the luminal and abluminal plasma membrane and diffusion of FA (FABP3 and FABP4) through the cytosol of ECs. This implies that studies that aim to determine transport rates from the lumen of the capillary into the skeletal muscle fibre should consider the role of the ECL as a potential barrier (limiting skeletal muscle uptake) or as a facilitator (exceeding or driving the muscle uptake; eg the ability of the endothelial insulin transport system to create a higher insulin concentration in the cytosol of ECs than in the lumen of capillaries and in muscle interstitium). This finding also implies that studies using extracts of muscle homogenates and Western blots to measure the content of these transporters cannot assume that the measured concentration reflects the protein content of the muscle fibres, if the transporter is expressed in a much higher concentration in the capillary endothelium than in the plasma membrane or cytosol of skeletal muscle. Examples of the latter are FAT/CD36 content (Vistisen *et al.* 2004) and FATP4 (Iso *et al.* 2013). Use of Western blot data in that case may confound important isomer differences between the capillary endothelium and the skeletal muscle fibres.

Another important message is that a careful review of earlier publications (Lund *et al.* 1987; Delashaw & Duling 1988) using intravital microscopy of red blood cells moving in the microvasculature of the anterior tibialis muscle of hamsters confirms Microfil® cast images of rat skeletal muscle (Fig 1C) of Segal (2005) suggesting that MVUs are the smallest functional elements that can be used to adjust capillary blood flow in response to physiological stimuli (insulin, exercise, adrenaline) and metabolic demand of muscle fibres. The blood flow patterns observed in these studies suggest that some MVUs have no flow, some undergo cyclic vasomotion and some are continuously perfused with a coordinated response occurring in each of these cases in all capillaries sharing a common TA. This pattern is not in line with recent suggestions that all capillaries in skeletal muscle are simultaneously perfused also at rest, but only over a fraction of their length with longitudinal recruitment occurring during exercise (Poole *et al.*, 2013).

The recent observation (Hagberg *et al.* 2010) that the VEGF-B gene is co-expressed in mouse skeletal muscle and brown adipose tissue with nuclear genes encoding for mitochondrial proteins and simultaneously has a unique role in the expression of FATP3 and FATP4 in the capillary endothelium of these tissues is important for the following reasons: 1) The observation seems to provide a mechanism for the fact that meal derived lipids in lean healthy and physically active humans are preferentially channeled for storage and subsequent oxidation into skeletal muscle and subcutaneous adipose tissue (Bickerton et al 2007); 2) As VEGF-B production similar to VEGF-A is likely to be lower (and less frequent) in sedentary individuals, the latter similar to VEGF (-/-) mice are likely to deposit meal derived lipids in ectopic fat stores (visceral adipose tissue, around blood vessels and in man probably also in the liver) and develop obesity. As sedentary and obese individuals have a low capacity for fat oxidation in skeletal muscle this may also explain the accumulation of FA metabolites (DAGs and ceramides) in their muscle, which is known to lead to insulin resistance (Fig. 3) and eventually to chronic disease (type 2 diabetes and cardiovascular disease). Future research should investigate these hypotheses by making comparisons between trained and sedentary humans and studying the impact of obesity, ageing and type 2 diabetes on the protein content of endothelial nutrient and insulin transporters. As VEGF-B controls the expression of FATP3 and FATP4 via VEGF receptor 1 and neuropilin 1 this research may also create opportunities for the development of novel pharmacological interventions to reverse the pathological metabolic consequences of obesity, ageing, type 2 diabetes and cardiovascular disease.

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Figure 1. Structure and anatomy of skeletal muscle microvasculature.

Panel A shows a cross-sectional image of muscle fibres and their microvasculature. It historically and today still is common practice to use cross-sectional images of skeletal muscle to visualise and quantify the capillarisation of skeletal muscle fibres. This image shows in green the plasma membrane of the skeletal muscle fibres and the membrane surrounding the ECL of capillaries (visualised with an antibody stain against collagen IV, which is a main component of the basement membrane). The glycocalyx of the capillaries and of the larger 4th order arteriole (approximately 50 μm in diameter) was stained in red with a fluorescent lectin (Ulex europaeus). This unpublished image comes from the collection of Dr Chris Shaw and was obtained by Chris during his PhD with Anton Wagenmakers at the University of Birmingham, UK; Panel B and C show microvascular casts in mouse gluteus maximus muscle. In these whole-mount preparations, the vasculature was cast using Microfil® and muscle fibres were cleared in glycerin to enhance the visibility of microvessels. Panel B shows that the arteriolar and venular networks are paired in skeletal muscle. A 3rd order arteriole (a) and its paired venule (v) both enter at left. Note the proximity of arteriolar-venular segments through the 4th order branches, which then branch into the TAs that supply red blood cells to the capillaries. The "shadows" of parallel muscle fibres and surrounding capillaries are oriented obliquely across the figure. Panel C shows that capillaries are organised into microvascular units (MVUs). TAs (arrowheads indicate 2 of the several that are present) branch off vertically from 4th order arterioles. Each TA gives rise to a group of approximately 20 capillaries. The capillaries in the MVU run half and half in both directions parallel to muscle fibres and have a length of approximately 1 mm. The capillaries converge on collecting venules (arrows indicate 2 of the several which are present). Muscle fibres are transparent in this image. Panel B and C are reproduced with permission of the

author and the publisher from Segal SS (2005). Regulation of blood flow in the microcirculation. *Microcirculation* **12,** 33-45. © John Wiley and Sons. Panel 1D is a visual representation of the relative size of the endothelial surface area in m^2 present in arteries, the microvasculature and veins. Every blood vessel in the human vasculature is covered on the luminal side with a continuous monolayer of ECs. About 400 m^2 ECL surface is present in skeletal muscle capillaries to generate the transport capacity for oxygen, fuels and hormones that is required to meet the high metabolic demands of skeletal muscle during aerobic exercise via recruitment of additional MVUs (compared to rest) and rhythmic increases in the dilatation of TAs and capillary blood flow in the recruited MVUs (compared to rest).

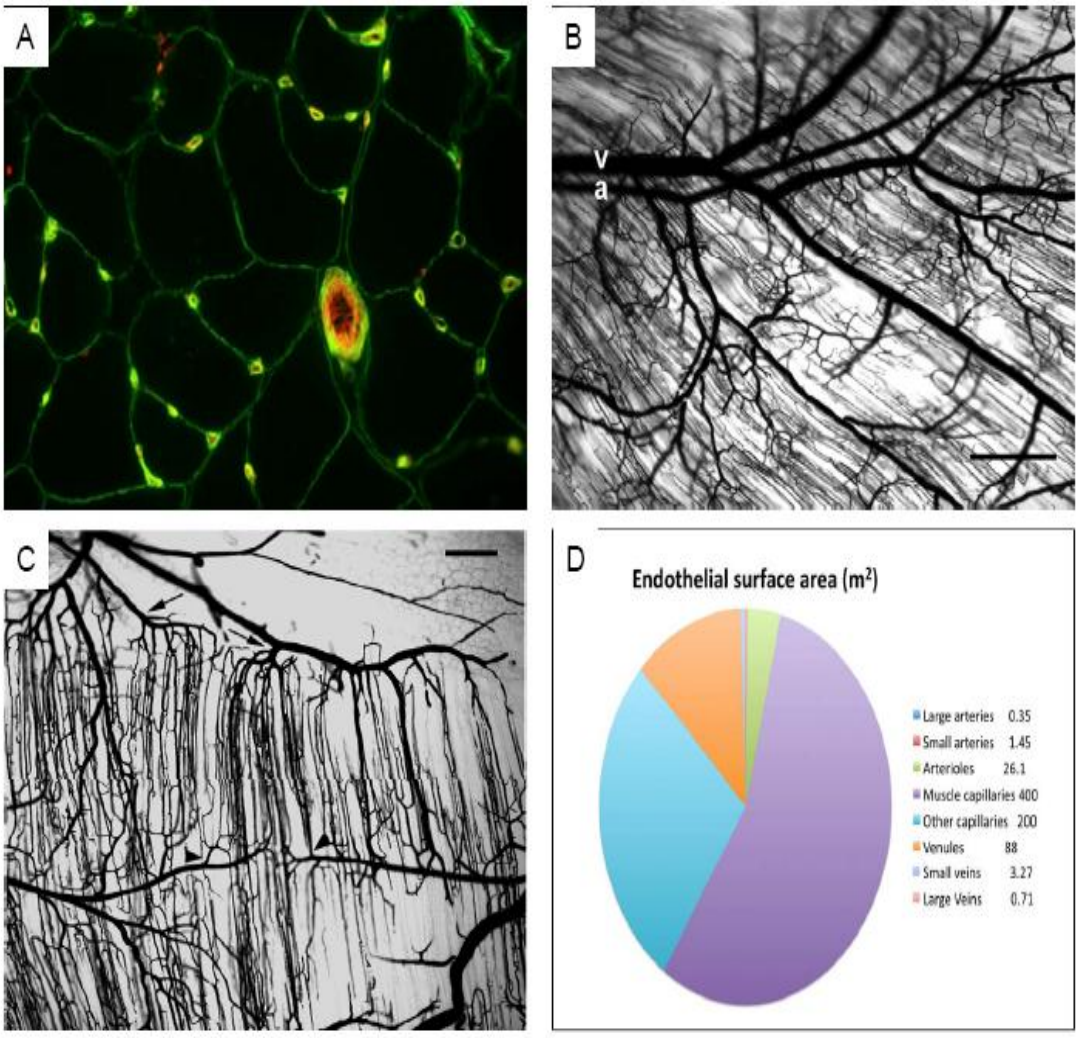

Figure 2. Insulin induced vasodilatation of TAs leads to increased perfusion of muscle capillaries.

Meal-induced increases in the plasma insulin concentration activate an insulin signalling cascade (Insulin receptor/IRS-1/PI3-Kinase/PDK1/Akt/eNOS), that is present in the ECL of the TAs. The endresult is an increase in nitric oxide (NO) production. NO is a potent vasodilator acting upon the smooth muscle cell layer in TAs. The simple version of the underlying mechanism is that insulin-induced increases in vasodilatation of TAs lead to recruitment of additional MVUs and capillaries that were not perfused before ingestion of the meal and, therefore, explain the observed increase in microvascular perfusion of skeletal muscle. The real mechanism probably is more complex as several studies have shown that the microvascular blood flow in skeletal muscle undergoes rhythmic oscillations attributable to spontaneous changes in the diameter of the TA lumen (Lund *et al.* 1987; Newman *et al.* 2009). This phenomenon is called 'arteriolar vasomotion'. Newman *et al.* (2009) using laser Doppler flowmetry recently observed that a hyperinsulinemic euglycemic clamp in rats increased the arteriolar oscillations. The interpretation of this observation by the authors is that the insulin-induced increase in microvascular perfusion of skeletal muscle is at least in part due to an increase in the intensity of the vasomotion that occurs in TAs of skeletal muscle. The left panel of Fig. 2 is an adaptation of Fig. 1 published in Cohen *et al.* (2000).

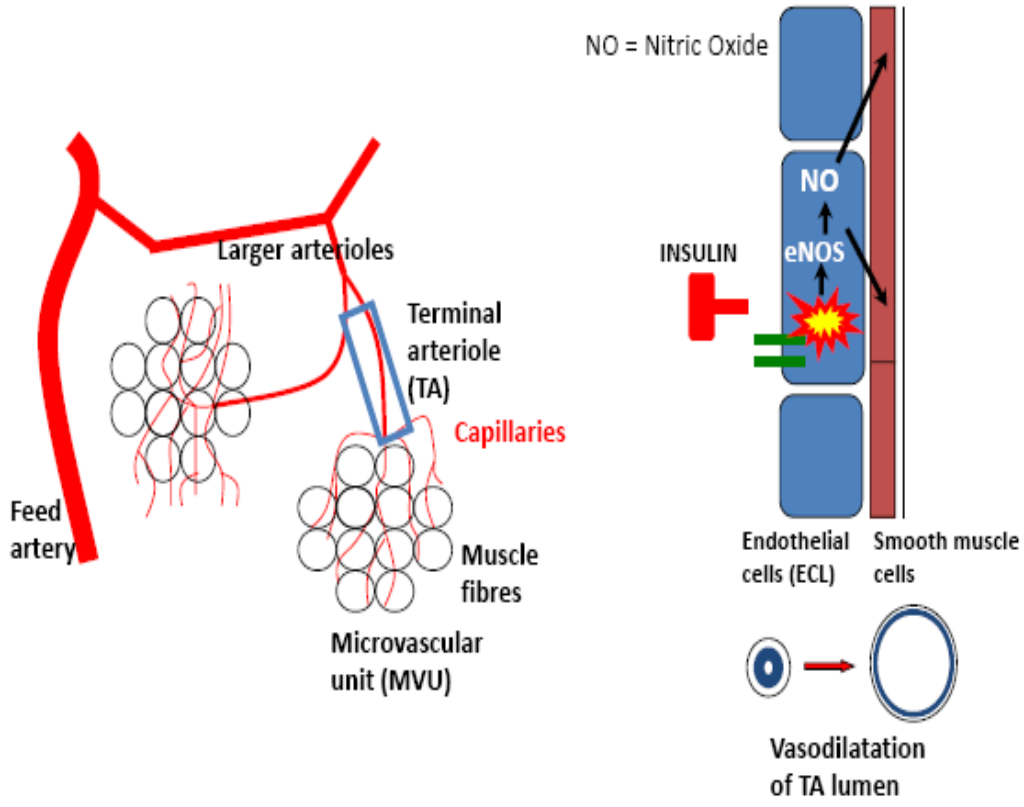

Figure 3. Activation of the insulin-signalling cascade in skeletal muscle.
In healthy trained individuals increases in the insulin concentration in the interstitial fluid surrounding muscle fibres leads to increased activation of the insulin signalling cascade and translocation of GLUT4 (main glucose transporter in muscle) to the plasma membrane. As muscle is the main tissue responsible for glucose uptake in the period after meal ingestion this will keep the rise in blood glucose concentration following ingestion of a meal relatively modest. As such healthy trained individuals have firm control on their blood glucose levels, both in the fasted period and after food intake. In sedentary, obese or elderly individuals and those with metabolic syndrome and type 2 diabetes high blood levels of fatty acids and triglycerides contribute to an excessive accumulation of lipid metabolites such as long chain fatty acyl-CoA, diacylglycerol and ceramide in skeletal muscle. This together with increased plasma levels of inflammatory cytokines in obese individuals (Berg & Scherer 2005) and local inflammation of the microvasculature in skeletal muscle activates the serine kinases PKC, IKK and JNK (Wagenmakers *et al.* 2006). These phosphorylate insulin receptor substrate-1 (IRS1) on serine residues leading to inactivation of IRS1 and downstream inactivation of the insulin signalling cascade. Ceramide accumulation also activates the phosphatase PP2A which dephosphorylates and inactivates Akt. These mechanisms collectively prevent activation of the insulin signalling cascade in skeletal muscle and, therefore, reduce insulin-mediated GLUT4 translocation and skeletal muscle glucose uptake. Reproduced with permission, from Shaw CS & Wagenmakers AJM, (2012), *The Biochemist*, **34,** 20-23. © the Biochemical Society.

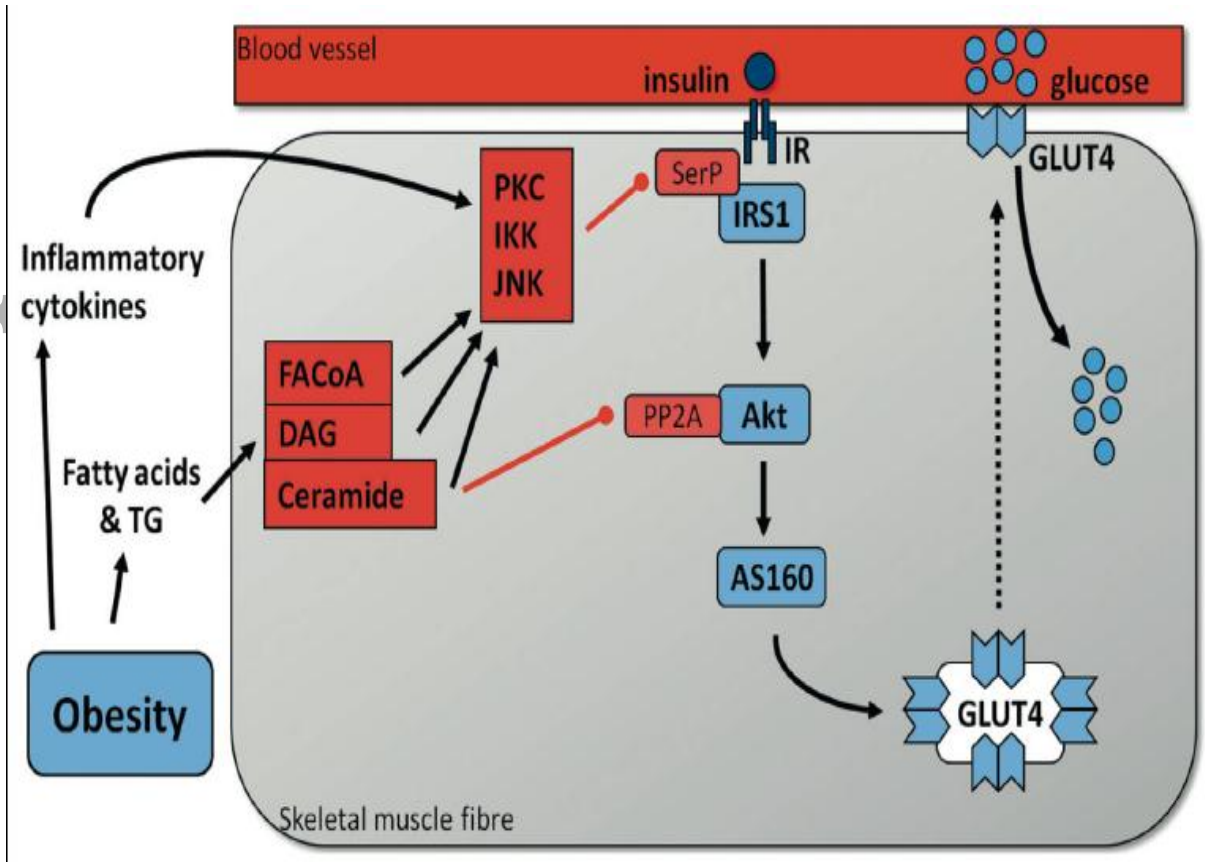

Figure 4. Schematic illustration of the role of VEGF-B in matching transendothelial FA transport to skeletal muscle FA oxidation capacity.

Hagberg *et al.* (2010) made the observation that VEGF-B in mouse skeletal muscle co-expressed in a large number of different physiological conditions with a large cluster of nuclear genes encoding for mitochondrial proteins. They subsequently generated strong supportive evidence for the existence of a novel mechanism, which couples uptake and transendothelial transport of FA to the capacity of mitochondrial β-oxidation in skeletal muscle. VEGF-B produced by skeletal muscle fibres was assumed to be released to the interstitium and then diffuse to the abluminal membrane of ECs in skeletal muscle capillaries, similar to the VEGF-A release by skeletal muscle during exercise (Hoier & Hellsten, 2014). Here, VEGF-B binds to its receptors (VEGF receptor 1 (VGRF1) and neuropilin 1 (NRP1)) which are both expressed by the ECs. Signalling of VEGF-B then leads to the abundant expression of FATP3 and FATP4 in the ECs and incorporation into the luminal and abluminal membrane and, therefore, is assumed to lead to increased TET of FAs into the muscle interstitium where FA are bound again to albumin to diffuse to the muscle plasma membrane and to be taken up and oxidised by skeletal muscle as described by Glatz *et al.* (2010). The red arrows in this Figure indicate the sequential steps in this novel mechanism as proposed by Hagberg *et al.* (2010). Other studies have shown that FAT/CD36 (Vistisen *et al.* 2004) and FABP4 and FABP5 (Iso *et al.* 2013) are also expressed abundantly in ECs and contribute to the high capacity for TET of FAs, but Hagberg *et al.* 2010 failed to generate evidence that expression of their genes was under the control of VEGF-B. It is also tempting to speculate that VEGF-B controls expression of the 1500 nuclear encoded genes in the ECs as the metabolic rate and protein turnover rate of ECs is high potentially because of repeated exposure to reactive oxygen species (ROS). It also cannot be excluded that some of the TET systems that help to increase endothelial permeability in the postprandial state and during exercise are energy (ATP) dependent (eg the insulin transport system which concentrates insulin in the ECs against a concentration gradient). This implies that ECs in trained inviduals should also have a high mitochondrial density to optimally support a high metabolic rate and the various transport functions.