Adaptation of Skeletal Muscle Microvasculature to Increased or Decreased Blood Flow: Role of Shear Stress, Nitric Oxide and Vascular Endothelial Growth Factor

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Key Words

Muscle activity · Ischaemia · Angiogenesis · Arterioles

Abstract

This review elucidates the roles of capillary haemodynamics, nitric oxide (NO) and vascular endothelial growth factor (VEGF) in the remodelling of skeletal muscle microcirculation in response to increased (electrical stimulation) or decreased (chronic ischaemia) blood flow. During early stages of stimulation-induced angiogenesis, up-regulation of VEGF and its receptor VEGF receptor 2 is dependent on shear stress and NO release, whereas later, involvement of NO in the expanding capillary bed appears to be VEGF/VEGF receptor 2 independent. Arteriolar growth most likely relies on mechanical wall stresses while growth factor involvement is less clear. By contrast, in muscles with restricted blood flow, increased VEGF/VEGF receptor 2 expression after ischaemia onset is not associated with changes in shear stress or hypoxia, or capillary growth. After several weeks, VEGF protein levels are lower than normal while modest angiogenesis takes place, a temporal mismatch that limits the utility of using growth factor levels during ischaemia to assess angio-

genic potential. Chronic stimulation of ischaemic muscles restores their depressed endothelial-dependent arteriolar dilatation, increases capillary shear stress and VEGF receptor 2 and promotes capillary growth. In patients with peripheral vascular disease, electrical stimulation of ischaemic calf muscles increases blood flow, capillary surface area and muscle performance, offering an alternative 'endogenous' treatment to gene or cell therapy.

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Introduction

The role of mechanical forces in the growth and remodelling of vessels was described more than 100 years ago by Thoma [1], who showed the importance of velocity of blood flow and/or pressure and of surrounding tissue expansion in the growth of vasculature in the chick embryo. Later, Clark [2] and Clark and Clark [3] demonstrated the role of flow in the increase and regression of capillaries during frog larvae development and rabbit ear chamber wound healing, respectively. Despite this work, relatively little is known about the importance of mechanical forces and the mechanisms of their transduction during the growth of vessels in normal adult tissues.

Skeletal muscles are one example where vessel growth occurs under physiological circumstances in the course of exercise training. The time scale and extent of such

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growth is dependent on the type of training and is related to the increase in blood flow in the active muscles. Endurance training involves oxidative muscles, increasing blood flow to this fibre type 5–6 fold and leading to associated capillary growth (15–20% increase in capillary: fibre ratio). In contrast, with high-intensity sprint training, blood flow is increased 3–4 fold in muscles composed of white glycolytic fibres and a 20% increase in capillary supply occurs specifically in relation to these fibres, whereas these parameters are not changed in oxidative muscles in this type of training [4–7]. The onset of capillary growth is dependent on the intensity of training and is much faster in animals trained by running to exhaustion [8]. Analysis of the individual factors contributing to capillary growth during whole body exercise is complicated by the fact that there are many central cardiovascular system alterations as well as changes in water balance, acid-base status and hormonal milieu. Indeed, vascular adaptation to whole body exercise can extend well beyond the active muscle groups [9]. In contrast, activation of a specific muscle group by electrical stimulation results in a similar onset and pattern of capillary growth as with exhaustive exercise [10] but without the confounding aspects described above. Such ‘involuntary’ local muscle activity provides a model whereby individual mechanical factors relating to muscle contraction and blood flow and their transduction mechanisms can be studied more precisely.

Repeated muscle contractions alter the local microcirculatory haemodynamics by dilatation of arterioles leading to increases in capillary flow velocity, shear stress and, potentially, pressure. At the same time, there are spatial changes in the configuration of capillaries with the shortening and lengthening of sarcomeres during each muscle contraction. We have previously studied the effects of high muscle blood flow per se (3-fold increase after treatment with α_1 receptor blocker prazosin), or stretch alone (lengthening muscle overload), or a combination of both types of mechanical stimuli (local muscle stimulation that causes contractions and doubles muscle blood flow) on the pattern of capillary growth [11] and described the involvement of metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) in capillary growth in these models [12]. In summary, capillary shear stress and wall tension are increased in muscles exposed to a long-term increase in blood flow, either by vasodilator treatment or stimulation, whereas there is no increase in blood flow [13] and a decrease in capillary flow velocity when muscles experience prolonged stretch [14].

Capillary growth in response to shear stress proceeds by division of the lumen by endothelial cell protrusion and vessel splitting, without the requirement for disturbance and breakdown of the basement membrane. MMP-2 mRNA and protein are inhibited/down-regulated by shear stress-induced nitric oxide (NO) release [15]. Stretch, on the other hand, leads to capillary growth by endothelial sprouting with increased MMP-2 expression [16], while in stimulated muscles, growth proceeds by both pathways.

Although the importance of VEGF for the maintenance of capillary supply in skeletal muscle has been demonstrated in VEGF-depleted mice [17], its expression in relation to mechanical stimuli of shear stress and/or NO in muscles exposed to long-term increases in activity has not been fully clarified. This review attempts to analyze the role of shear stress in the activation of VEGF and its receptors and/or NO in skeletal muscles with growth (training induced) or regression (restricted blood supply) of capillary and arteriolar microcirculation. It also emphasizes the role of different factors in the initiation of angiogenesis and subsequent microvascular remodelling (muscles stimulated for 2–4 or for 7 days), as well as in the early (1–3 days) and late (up to 35 days) stages of ischaemia-induced microvascular adaptations.

Adaptation of the Microvascular Bed to Increased Blood Flow

Growth of Capillaries

There is now a considerable body of evidence linking beneficial adaptation of large conduit and resistance arteries during exercise training with endothelial effects of shear stress [9, 18]. In contrast, although capillary growth and blood flow in trained or ischaemic muscles have been studied for decades, there are few data on capillary shear stress under these conditions. It is of course known, on the basis that capillary red blood cell velocity increases significantly during acute muscle contractions [19–21], that shear stress will be elevated [22]. In muscles exposed to increased activity by stimulation for 8 h/day for 2 days, capillary shear stress was doubled even when muscles were at rest [23]. Also at this time, proliferation of capillary endothelial cells positive for proliferating cell nuclear antigen was raised 4.5 fold [10], but the number of capillaries was not yet increased. After a further 5 days of stimulation, shear stress and red cell velocity in individual capillaries had returned to control values since there were many more pre-capillary arterioles (density in-

Fig. 1. Schematic representation of the time course of changes in microcirculatory haemodynamics, growth factor expression and angiogenesis during stimulation-induced muscle activity. Arrows represent increases relative to control inactive muscles. C:F ratio = Capillary:fibre ratio.

creased by 112%) distributing flow through a capillary bed that had almost doubled in size [24].

It is known that increased shear stress in endothelial cell cultures leads to an increase in protein expression of VEGF receptor 2 (Flk-1) [25]. A similar increase was seen in skeletal muscles of mice where capillary growth was induced by long-term administration of prazosin [26] and in stimulated rat muscles, although no comparable change was found in the expression of VEGF receptor 1 (Flt-1) [27]. The time course of these changes revealed that the effect of shear stress on VEGF receptor 2 was greatest in muscles stimulated for 2 days (protein expression $2.5 \times$ control) and waned at 7 days ($1.2 \times$ control), demonstrating a clear association with levels of shear stress in individual capillaries. In contrast, expression of the ligand VEGF increased by 50% after 2 days of stimulation and remained elevated throughout the period of muscle activity [10]. VEGF protein is located in the subsarcolemmal region of skeletal muscle fibres and in vascular smooth muscle in addition to the capillary wall [28,

29], the latter site being the most likely to exert an effect on endothelial cell proliferation. Immunohistochemical detection of VEGF protein revealed no change in the proportion of VEGF-positive capillaries after 2 days of stimulation, but a substantial increase from 15% in control muscles to 45% in muscles stimulated for 4 and 7 days [28]. Based on these findings, a possible sequence of events in capillary growth in this model of activity-induced angiogenesis is presented schematically in figure 1.

NO is known to be released by shear stress, and its relationship to the regulation of VEGF during angiogenesis has been studied both in vitro and in vivo [30, 31]. It is therefore important to evaluate the role of NO in activity-induced skeletal muscle angiogenesis. The expression of endothelial NO synthase (eNOS) protein was increased in muscles after 2 days of stimulation [10], and with longer duration, the expression of neuronal (n)NOS was elevated [32]. When eNOS and nNOS activity in stimulated muscles was inhibited by daily administration of N^{ω} -

nitro-L-arginine, the increase in capillary shear stress observed after 2 days of stimulation was abolished, and shear stress was actually decreased in muscles stimulated for 7 days [23]. NOS inhibition eliminated the increased expression of VEGF and VEGF receptor 2 protein in the early stages of stimulation (2–4 days) but was without effect on their expression by the later stage (7 days). NOS inhibition also prevented capillary proliferation and the increase in capillary:fibre ratio in stimulated muscles [10]. Thus, increased capillary shear stress appears to induce the release of NO and increase VEGF and its receptor 2 expression, either directly or via NO, during the initiation of endothelial cell proliferation and angiogenesis. The later stages of capillary growth are more likely modulated by NO independently of the VEGF cascade, possibly via extracellular regulated kinases ERK-1/2 [33] or by activation of protein kinase C, ERK and c-Jun [34]. The involvement of NO in shear stress-mediated angiogenesis is also corroborated by the fact that NOS inhibition prevented capillary growth induced by increased blood flow in the chronic vasodilator treatment model, but not in muscles exposed to mechanical stretch [35] in which the initial stimulus leads to metalloproteinase activation, breakage of basement membrane and endothelial sprouting.

Growth of Arterioles

An increase in total blood flow through an exercise-trained muscle will depend not only on expansion of the capillary bed but also on growth of the pre-capillary arteriolar vessels that regulate flow through each group of capillaries [36] as well as the expansion of the upstream resistance vasculature. However, there is less information about the growth of arterioles than of capillaries in trained muscles, but it appears that, unlike capillary growth, there is no direct relationship with blood flow in the active muscles. A higher density of small arterioles has been reported after training by running, but this occurred in the rat spinotrapezius muscle that was not activated during the training exercise [37]. Moreover, endurance training in rats led to increased arteriolar density in both the white and red part of the gastrocnemius, in contrast to oxidative fibre-specific capillary growth [38], and a similar result was achieved by intensive sprint training [39]. Muscle activity induced by stimulation increased the number of pre-capillary arterioles with a similar time course to the growth of capillaries [24]. Arteriolar growth has also been reported in muscles following chronic va-

sodilator treatment [40] and hypoxia [41], but it is not known whether this is directly related to blood flow and shear stress. Growth of pre-capillary arteriolar vessels is thought to occur by apposition of smooth muscle cells or pericytes to newly formed capillaries [42], and it has been suggested that the stimulus is pressure or circumferential wall stress rather than increases in shear stress [43]. Despite the importance of arterioles as flow controllers, the role of individual mechanical factors and any interaction between them [44], in determining arteriolar as opposed to capillary remodelling, is far from clear.

Adaptation of the Microvascular Bed to Decreased Blood Flow

Capillary Remodelling in Ischaemia

Changes in capillary and arteriolar supply have been studied in different animal models of ischaemia that are intended to mimic the clinical conditions of peripheral vascular disease [45]. In many cases, these involve excision of substantial arterial segments such that profound ischaemia is caused, leading to severe muscle damage, degeneration and necrosis. This is usually followed by regeneration and marked angiogenesis that is mediated by growth factors and cytokines accompanying the inflammatory state [46, 47], facilitated by dynamic regulatory systems such as angiopoietin-Tie ligand signalling [48]. Whilst this sequence of events initially replicates the condition of critical limb ischaemia, it is not representative of patients with less severe atherosclerosis who may have normal lower limb blood flow at rest but impaired functional hyperaemia. These individuals experience intermittent claudication, typically on exercise, but do not show signs of ischaemic inflammation or any significant angiogenesis [49, 50]. The capillary:fibre ratio may even be reduced in long-standing mild-to-moderate peripheral arterial disease [51]. This latter condition is more closely mimicked by ligation of a single artery, e.g. iliac or femoral, and, in the rat, ligation promptly reduces limb blood flow both at rest and, most notably, during muscle contractions to 16% of normal. However, this is not sufficiently severe to cause necrosis and inflammation, and there is no macrophage infiltration [52] or other sign of muscle damage [53] and, moreover, no immediate capillary growth. Over the course of 2–5 weeks, the development of collateral circulation gradually restores flow to near normal levels, and it is only at this time that a remarkably modest degree of angiogenesis occurs, preceded by capillary endothelial proliferation [27, 52].

From the previous section, it is evident that increased shear stress can up-regulate the expression of VEGF in capillaries. However, VEGF production is also known to be stimulated by hypoxia [54], and its enhanced expression has been observed in skeletal muscles that are severely ischaemic and undergo inflammatory regeneration [46]. As such, it has been widely viewed as a key mediator of ischaemia-induced angiogenesis in muscle [55], activated via hypoxic transcription factor HIF-1 α or HIF-independent transcriptional coactivator PCG-1 [56]. During the development of mild-to-moderate ischaemia in the model of iliac artery ligation, resting blood flow decreased to 30–40% of the control values after 3 days and, although it gradually increased, was still lower than in controls 5 weeks later. Blood flow during acute contractions did not increase in ischaemic muscles whereas in normally perfused muscles it increased more than 9 fold. The pattern of VEGF mRNA and protein expression was followed for up to 5 weeks after ligation, both showing a biphasic response. VEGF mRNA increased more than 7 fold during the first 3 days, returning to control level after 7 days, and increasing again to 1.5–2 times the control levels in the later stages. VEGF protein expression was elevated by 50% at the early time points, normalized after 7 days, and was actually lower than in controls at the time when proliferation and growth of capillaries occurred. Taken together, VEGF mRNA and protein expression, although elevated at the point of peak ischaemia and hypoxia, were attenuated over time, demonstrating that assessment of levels of this growth factor might not be as useful an index of angiogenic potential in response to ischaemic insult as previously thought [57].

Ischaemia by femoral artery ligation in mice also resulted in increased expression of VEGF and VEGF receptor 2 proteins, but only in a slight increase in HIF-1 α and in no significant changes in capillary supply in lower limb muscles. Angiogenesis occurred only when the activity of HIF-1 α was enhanced by an oxoglutarate analogue [58]. Thus, in mild-to-moderately ischaemic muscles, the late onset of capillary growth [27, 52] that is unrelated to HIF-1 α and does not coincide with the peak increases in VEGF mRNA or protein implies that hypoxia is not the main stimulus for capillary growth in this model.

On the other hand, capillary:fibre ratio and functional hyperaemic blood flow were both significantly increased in ischaemic rat muscles by the vasodilator prazosin administered for 2–5 weeks after ligation [59]. This suggests that restoration of blood flow and capillary shear stress in ischaemic muscles is important in the instigation of angiogenesis, a notion that is supported by the ap-

pearance of capillary proliferation concurrent with the development of collateral circulation and recovery of perfusion following iliac artery ligation in the rat [52]. In order to study more clearly the roles of shear stress and growth factors in ischaemic remodelling of the microcirculation, we created a model of more consistent ischaemia by ligation of the femoral artery 3 weeks after the iliac ligation (double ligation, DL), so as to abrogate the effects of the developing collateral circulation. This resulted in greater disturbance to capillary haemodynamics, with a higher proportion of capillaries having intermittent or no red blood cell flow. In contrast to normally perfused muscles where capillary shear stress increased by 50% following muscle contractions, shear stress did not increase at all in DL muscles [60]. Intermittent electrical stimulation applied for 2 weeks to ischaemic muscles was shown previously to improve their blood flow [61], and when DL muscles were stimulated in this way, the percentage of continuously flowing capillaries became even higher than in control muscles and there were no capillaries without flow. Post-contraction capillary shear stress was 2.8 times that at rest, and it exceeded that in control muscles by 50%.

Following double ligation and more persistent ischaemia, levels of VEGF protein were increased 4 fold, but there was no change in VEGF receptor 2 expression and no capillary growth. Application of intermittent stimulation to these DL muscles significantly enhanced VEGF receptor 2 expression, as in normally perfused muscles after stimulation, and, although VEGF protein levels were somewhat reduced, resulted in proliferation of capillary endothelial cells and increased capillary:fibre ratio [27]. This emphasizes yet again that up-regulation of VEGF alone is not necessarily sufficient to elicit angiogenesis, and that significant capillary growth occurs in muscles with prolonged ischaemia only in association with stimulation-induced increases in capillary shear stress and enhancement of VEGF receptor 2 expression that exceed levels in both control and ischaemic muscles (fig. 2).

Arteriolar Remodelling in Ischaemia

There is a wealth of literature concerning the development of collateral circulation in ischaemic models, much of it focusing on ‘arteriogenesis’, the growth and expansion of pre-existing vasculature proximal to the ischaemic territory to create a natural by-pass. Since this remodelling takes place away from the distal hypoxic tis-

Fig. 2. Schematic representation of the time course of changes in microcirculatory haemodynamics, growth factor expression and angiogenesis during chronic mild-to-moderate muscle ischaemia induced by arterial ligation. Arrows showing changes resulting immediately after ligation and during early and late ischaemia represent alterations relative to control non-ischaemic muscles. With application of activity by stimulation to ischaemic muscles, arrows represent changes relative to both control and ischaemic muscles. C:F = Capillary:fibre ratio.

sues, Schaper and collaborators [62, 63] have summarized the factors stimulating arteriogenesis as the physical forces of fluid shear stress and pressure, in conjunction with cytokines and chemoattractants that aid the cell proliferation and incorporation of new elements required for vessel enlargement. In contrast, within the ischaemic muscles, impairment to red cell perfusion in capillaries is initially due to the reduced perfusion pressure, and this, over time, leads to marked changes in the reactivity of pre-capillary arterioles that exacerbate this situation. In both our models of ischaemia, iliac artery and double ligation, endothelial-dependent dilation of these flow-

controlling arterioles was lost early on, with later deterioration in constrictor reactivity [60, 64]. These functional impairments clearly impact upon capillary perfusion, and, when DL muscles received intermittent stimulation, endothelial-dependent and -independent dilation were both restored, contributing to substantial increases in capillary shear stress [60]. These effects likely involve NO because the loss of arteriolar reactivity could be mimicked by chronic NOS inhibitor treatment in normally perfused muscles and restored by stimulation [65].

Evidence concerning structural remodelling of these small arterioles during chronic ischaemia is conflicting.

Recently, Bailey et al. [66] reported an increase in the density of small arterioles in ischaemic mouse spinotrapezius muscle without signs of muscle hypoxia, but in this small muscle, the pressure gradients following ligation would be different from our rat models where ligation is further upstream and hence more distant from the ischaemic muscles investigated. Our own studies have shown a significant reduction in α -smooth muscle actin staining of pre-capillary arterioles 10–20 μm in diameter, suggesting a loss of smooth muscle cells possibly due to the low flow and pressure condition, as well as a reduction in total eNOS protein expression [52, 65]. On the other hand, this loss of smooth muscle cells could be mimicked by NOS inhibition, which elevated perfusion pressure but impaired dilator function [65]. In both situations, i.e., ischaemia and NOS inhibition, intermittent stimulation restored arteriolar reactivity and resulted in significant increases in α -smooth muscle actin-positive arteriolar density, suggesting that re-establishment of flow in arterioles is important for their structural integrity. As chronic stimulation also increased the expression of eNOS protein [60] and L-arginine treatment was able to restore arteriolar density in ischaemic muscles [67], NO may play a role, possibly by aiding the recruitment of mural cells [68]. In light of recent work showing either increase [69, 70] or no change [71, 72] in α -smooth muscle staining in ischaemic muscles of patients with long-lasting peripheral arterial disease, the adaptation of arterioles to ischaemia will need much more detailed investigation.

Application of Stimulation Treatment in Peripheral Arterial Disease

It is well known that exercise training improves many symptoms in patients with peripheral vascular diseases [73, 74] but adherence to training programmes is often limited by co-existing cardiovascular disease, or the programme has to be adjusted to suit the limitation to exertion by the worst affected leg. Use of local muscle electrical stimulation applied to one leg at a time avoids these constraints and provides a feasible surrogate exercise alternative. When patients with intermittent claudication, limited to pain-free and maximum walking distance of about 50 and 100 m, respectively, stimulated their calf muscles for 20 min 3 times each day for 4 weeks, their pain-free walking increased by 82% and their maximum walking by 44% [75]. Their calf muscle fatigue during isometric contraction, very much reduced by ischaemia, be-

came similar to age-matched controls [75, 76]. The microvascular benefits of stimulation were evident as a 44% increase in calf blood flow and a significant increase in capillary filtration coefficient [77].

Growth factor gene therapy and stem cell treatment are currently undergoing clinical trials as promoters of neovascularization in peripheral vascular disease but have so far yielded inconsistent results as to therapeutic effectiveness [78]. In part, this is due to difficulties in harnessing and coordinating the complexities of cellular and molecular mechanisms that lead to the addition of mature fully functional vessels particularly in critically ischaemic inflammatory conditions. Electrical stimulation offers a simple alternative means to recruit endogenous systems whereby angiogenesis can be achieved and the microvasculature of ischaemic muscle enhanced.

Conclusions

Muscle activity increased by chronic electrical stimulation leads to the enlargement of the microvascular bed, demonstrated as increased numbers of capillaries and arterioles. Capillary shear stress, increased as a result of this activity, plays an important role in activating NO, VEGF and VEGF receptor 2 in the early stages while NO, released probably by activation of nNOS, is important in the later remodelling. Shear stress due to increased capillary perfusion is also important in inducing capillary growth in ischaemic muscles, possibly by activation of VEGF receptor 2. Electrical stimulation can be used as an effective treatment in patients with peripheral vascular disease by increasing their muscle blood flow and capillary surface area.

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