Association between Shear Stress, Angiogenesis, and VEGF in Skeletal Muscles

In Vivo

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ABSTRACT

Objective: To investigate the hypothesis that capillary proliferation in skeletal muscles, induced by a long-term increase in blood flow which elevates capillary shear stress, is associated with capillary expression of vascular endothelial growth factor (VEGF).

Methods: Adult rats received prazosin in drinking water (~2 mg per day) or had extensor digitorum longus (EDL) muscles stimulated by implanted electrodes for up to 14 days. At intervals, serial frozen sections of EDL were stained for alkaline phosphatase to identify capillaries, proliferating cell nuclear antigen (PCNA), and VEGF-A protein. Shear stress was estimated from capillary red blood cell velocities and diameters, measured by direct observation of epi-illuminated EDL.

Results: Chronic stimulation and prazosin treatment both increased capillary: fiber ratio by ~40% after 14 days. In stimulated muscles, the percentage of capillaries positively stained for VEGF increased within 3 to 4 days, while the density of PCNA-positive capillaries had increased 20-fold after 2 days. With prazosin, VEGF-positive capillaries increased after 2 and 4 days, accompanied by a threefold increase in PCNA. By 14 days, PCNA labeling and VEGF were still high in stimulated muscles, but no longer different from controls with prazosin. After 3 to 4 days of treatment, capillary shear stress in resting muscle was 57% higher than in controls as a result of stimulation, but 4 times higher with prazosin.

Conclusions: Higher capillary shear stress with prazosin than with stimulation may upregulate VEGF expression in the early stages of treatment. Greater proliferation of capillaries preceding a higher proportion of VEGF-positive capillaries in stimulated muscles, in the presence of a modest increase in shear stress, suggests that angiogenesis was initiated by other factors in addition to shear stress.

INTRODUCTION

Increasing muscle activity by exercise training or indirect electrical stimulation results in substantial capillary growth (34). It has been suggested that this may be initiated by the prominent angiogenic vascular endothelial growth factor (VEGF), on the basis that VEGF mRNA was increased by as little as 1 hour of electrically evoked muscle contractions (9,43) or exercise (25). VEGF mRNA was also elevated in chronically stimulated muscles prior to (31) or concurrently with (47) the increase in capillarity. Annex and colleagues (4) reported elevation of VEGF protein in muscles stimulated for 3 to 56 days and demonstrated increased VEGF immunostaining of endothelial cells, although this was not quantified.

KEY WORDS: capillary growth, electrical stimulation, immunohistochemistry, prazosin, proliferating cell nuclear antigen (PCNA), red blood cell velocity, VEGF-A

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Muscle activity elevates blood flow, and the associated increase in microvascular shear stress is one factor that may initiate growth of capillaries. This hypothesis is supported by the fact that shear stress induces endothelial cell proliferation in vitro (3), and by reports of increased capillarization in skeletal and cardiac muscles in response to long-term administration of vasodilators (see Ref. 34). However, Roca and colleagues (43) did not find higher expression of VEGF mRNA in muscles to which blood flow was increased artificially for 1 hour to a level found during muscle contractions, whereas it did increase after long-term treatment for several weeks with the vasodilator prazosin (16,17,51). The hemodynamic factor common specifically to expression in relation to capillary endothelium. In fact, the source of VEGF in skeletal muscle has not been clearly identified, although it could occur in skeletal muscle fibers (9), smooth muscle cells, endothelial cells, or originate from other cell types such as macrophages (7) or fibroblasts (48).

Our previous data clearly demonstrated capillary growth in muscles after either chronic stimulation or treatment for several weeks with the vasodilator prazosin (16.17.51). The hemodynamic factor common to both these situations is increased blood flow, which ultimately leads to an elevated capillary shear stress (18), and possibly higher wall tension. High shear stress has been shown to be important in the upregulation of some growth factors in vitro (see Ref. 14). It results in endothelial cell reorganization and, possibly, disruption of the intercellular junctions, which triggers upregulation of VEGF mRNA and protein in bovine aortic endothelial cells (13). Based on these findings, we hypothesized that increased capillary shear stress may activate VEGF and participate in induction of capillary proliferation. We therefore studied the time course of capillary proliferation, expression of VEGF-A protein specific to capillaries (because its localization in relation to angiogenesis in skeletal muscle is not clear), and measured shear stress in skeletal muscles in which blood flow was chronically increased by muscle contractions or by vasodilation. Particular emphasis was placed on the changes occurring prior to an increase in the anatomical capillary supply.

MATERIALS AND METHODS
Animal Models

All experiments were performed on Sprague-Dawley rats, final body mass 230–320 g, in accordance with the UK Animals (Scientific Procedures) Act of 1986. Chronically stimulated animals had stainless steel, multistranded, Teflon-insulated electrodes implanted unilaterally in the vicinity of the peroneal nerve under fluothane anesthesia, as described in detail previously (16), and fixed externally to a connector on the skin. Animals received analgesic (Temgesic, 0.2 mL s.c.) before the end of operation, in the evening and twice during the first postoperative day. They were caged individually, and approximately 20 hours after operation were connected to a stimulator (Neurotech, Shannon, Ireland) to activate lower leg flexor muscles at 10 Hz, 0.3 msec pulse width, and voltage up to 5–6 V for 8 hours per day. Stimulation elicited strong contractions but did not cause the animals discomfort, as normal sleep pattern and weight gain were unaffected. The alpha-1 receptor antagonist prazosin (gift of Pfizer; Sandwich, UK) was administered to another group of individually caged animals in drinking water (50 mg/L); daily intake of the drug was estimated to be ~2 mg on the basis of volume consumption.

Chronic electrical stimulation and prazosin treatment were continued for a maximum of 14 days, and subgroups of animals from each treatment were taken into experiment at intervals prior to and including this time point. After stimulation, 3 animals in each subgroup were taken for muscle histology at
In Vivo Measurements

All animals were anesthetized by sodium pentobarbital (50 mg/kg i.p.) approximately 16 hours after the last period of stimulation or 4 hours after last consumption of prazosin, and a cannula inserted into the jugular vein for supplementation of anesthetic. In prazosin-treated rats, arterial pressure was measured via a cannula in the carotid artery. This was not done in the stimulated rats, as we have shown previously that there were no differences in blood pressure among the different groups of stimulated animals (20).

Capillary shear stress was estimated in muscles before capillary-fiber ratio was increased (i.e., after 4 days of prazosin treatment and 3 days of stimulation) and in control animals. These rats were prepared within 2 hours of anesthetic induction for direct observation of capillary diameters and red blood cells velocities, so that measurements were made in resting muscle at least 18 hours after the end of stimulation and 6 hours after last intake of prazosin. Extensor digitorum longus muscles (EDL) were exposed as described previously (2) and superfused by deoxygenated Krebs-Henseleit solution (131.9 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO4·7H2O, 2.0 mM CaCl2·2H2O, and 22.0 mM NaHCO3; pH 7.35–7.45, ~32 °C with a flow rate of 5 mL/min. Muscles were epi-illuminated and the surface observed under the intravital microscope (model ACM, Zeiss, Oberkochen, Germany) using a ×25 magnification water-immersion lens (0.6 n.a., Leitz). Images were displayed via a video camera (Cohu 4712) in real time on a television monitor at a final magnification of approximately ×1000 and stored to tape on a video recorder (Panasonic NV-F70HQ).

EDLs were excised and the mid portion, ~5-mm thick, was rapidly frozen in isopentane precooled in liquid nitrogen. Muscles were stored at −80 °C for later processing.

In Situ Hybridization. Based on rat VEGF164 sequence from Genbank (Accession No. M32167), oligonucleotide DNA probes were synthesized (Alta Bioscience, Birmingham, UK) as follows: 1) an antisense 27-mer probe complementary to positions 334–361, designated as vegf1: 5-CAT CTC TCC TAT GTC CTG CCT TTG GTG-3'; 2) an antisense 27-mer probe complementary to positions 470–496, designated as vegf2: 5-GCA GGA ACA TTT ACA CGT CTG CGG ATC-3'; and 3) a 24-mer oligoprobe complementary to the second probe VEGF2, designated as sense probe. VEGF and sense oligoprobes were 3' end-labeled with dioxigenin-11-dUTP (Dig Oligonucleotide Tailing Kit, Boehringer, Mannheim, Germany). To increase the sensitivity of hybridization, a cocktail of the two antisense probes, vegf1 and vegf2, was applied to each section. 10-μm frozen sections of EDL were fixed in 4% paraformaldehyde in PBS for 15 minutes, digested with proteinase K (1 μg/mL, Boehringer, Mannheim) at 30 °C for 30 minutes, washed in PBS, and air dried. Hybridization with 0.5 μg/mL of DIG-labeled sense or antisense VEGF oligoprobes was carried out at 38 °C for 16 hours. The posthybridization washes and immunological detection of the probes were performed according to standard protocol (8).

Immunohistochemistry. Three consecutive 6-μm sections from each muscle were stained sequentially 1) by anti-VEGF antibody, 2) for alkaline phosphatase (ALP) activity in capillaries using FAST BCIP/NBT tablets (Sigma, UK), and 3) by antiproliferating cell nuclear antigen (PCNA) antibody. For VEGF-A immunodetection, sections were fixed in cold (4 °C) acetone for 5 minutes. To block endogenous peroxidase activity and nonspecific binding, sections were incubated in 3% hydrogen peroxide
and pretreated with 10% normal horse serum. They were then incubated with 1:500 solution of anti-VEGF monoclonal antibody (mouse IgG, epitope corresponding to amino acids 1–140 of human VEGF, which cross-reacts with rat VEGF; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, and horse antimouse secondary antibody was applied at 1:200 for 1 hour. Bound antibody was detected using avidin–biotin immunoperoxidase method (ABC Kit, Vector, UK) for 30 minutes. The slides were exposed to 3,3 diaminobenzidine (DAB) to obtain a brown reaction product.

For PCNA detection, dried fixed (3% buffered formalin) sections were incubated in 3% hydrogen peroxide in methanol (5 minutes) to inhibit endogenous peroxidase. Nonspecific binding was blocked with a 1:200 solution of normal horse serum (Vector, UK) in PBS. Primary monoclonal mouse antibody against PCNA (clone PC10, Dako Ltd, UK) was applied at 1:100 for 1 hour at room temperature. After triple washing in PBS, sections were covered with 1:200 solution of biotinylated horse antimouse antibody (Vector, UK) for 1 hour, followed by ABC and DAB as above. Counterstaining with Mayer’s hematoxylin for 5 minutes enabled visualization of all cell nuclei.

As the very high density of proliferating cells previously observed in 2-day-stimulated muscles (35) could indicate a degree of inflammatory response and a potential source of VEGF, we also performed staining for ED1 as a marker of invasive macrophages. 6-μm sections from EDL were fixed in buffered formalin and incubated overnight at 4 °C with mouse monoclonal antibody antirat ED1 (Serotech, UK), diluted 1:500 in PBS. Subsequent steps were identical to the protocol for PCNA.

Quantification of Capillary Supply, Cell Proliferation, VEGF, and Macrophages

Serial sections were examined in four fields at either ×200 or ×400 (each 0.063 mm² at ×400) using an Olympus BFS-2 microscope equipped with a drawing arm to enable viewing of the same area in all three sections (Fig. 1). Capillary supply was evaluated as capillary:fiber ratio (C:F) from counts of capillaries and muscle fibers. The density of nuclei stained positively for PCNA was estimated and, in relation to the location of capillaries depicted by ALP, identified as being colocalized to capillary sites (PCNA cap/mm²) or situated in the interstitium outside capillaries (PCNA int/mm²). No PCNA-positive nuclei were seen in skeletal muscle fibers. Discrete loci of VEGF-positive immunostaining were observed, some of which could be colocalized to capillaries (Fig. 1), and some of which were associated with noncapillary interstitial cell types or inside muscle fibers in a perinuclear position (Fig. 2). The densities of VEGF-positive capillary-linked sites (VEGF cap/mm²) and noncapillary sites, including both interstitial and muscle subsarcolemmal locations, (VEGF noncap/mm²) were estimated. To evalu-
ate the specific relationship of VEGF to endothelial cells, the proportion of total capillaries present (ALP stained) that expressed VEGF protein was calculated. The density of cells stained by antibody for ED1 (monocytes and macrophages) was also estimated.

Statistical Analysis

All data are presented in the text as means ± SEM. Comparisons within treatments of time-dependent effects were made by repeated-measures ANOVA. Comparison between treatment effects were made by
factorial ANOVA with Fisher’s PLSD post-hoc test. Significance was set at 5%.

RESULTS

Capillary Supply and Cell Proliferation

Capillary supply was expressed as C:F ratio, as this parameter is relatively independent of muscle fiber area. Both prazosin and stimulation significantly increased C:F after 7 days, with slight further elevation over the following week (Table 1). The density of proliferating capillary-linked nuclei (PCNA_{cap}/mm^2) was similar in control muscles for prazosin and stimulation (Table 1). After 2 days, before new capillary growth was apparent, PCNA_{cap}/mm^2 showed a far greater increase in stimulated muscles (20-fold vs. control) than in those treated with prazosin (4- to 5-fold vs. control). With prazosin treatment, the more modest increase in PCNA-labeled capillaries after 2 days was maintained at 4 and 7 days and declined to values not significantly different from controls after 14 days. With stimulation, PCNA labeling colocalized to capillaries gradually declined after 2 days but was still more than 8-fold higher than controls after 14 days (Table 1).

In contrast, the density of noncapillary interstitial proliferating nuclei (PCNA_{int}/mm^2) did not change from control levels throughout the whole period of prazosin treatment. In stimulated muscles, however, there was a significant increase in interstitial PCNA labeling, which peaked after 7 days of stimulation and was back to control values after 14 days (30).

Expression of VEGF mRNA and Protein in Skeletal Muscles

Figure 2 shows an example of sections from 7-day-stimulated muscle hybridized with antisense [Fig. 2(A)] and sense [Fig. 2(B)] VEGF probes. Sites positive for VEGF mRNA are located mainly within the interstitial space, and some can be seen to be positioned at likely capillary sites. In addition, VEGF mRNA is present within the sarcolemma of muscle fibers. Figure 2(C) shows examples of the different locations of VEGF-A immunostaining that were observed, either situated at capillary sites [compare Figs. 2(C) and 2(D)] or localized to other cell types lying between muscle fibers, or within the muscle fiber in a perinuclear situation. Specificity of VEGF immunostaining was confirmed by the fact that it was totally abolished by preincubation of the anti-VEGF antibodies with VEGF peptide (Santa Cruz) in 1:10 ratio for 1 hour at room temperature [Fig. 3(B)], and the present quantitative analysis distinguished VEGF immunostaining at capillary sites from that in the interstitium and within muscle fibers.

Table 1. Capillary supply, capillary- and interstitial-associated cell proliferation, and VEGF expression, both capillary-associated and noncapillary

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
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<tr>
<td><strong>Prazosin</strong></td>
<td></td>
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<tr>
<td>C:F</td>
<td>1.45 ± 0.08</td>
<td>1.42 ± 0.11</td>
<td>1.62 ± 0.04</td>
<td>1.83 ± 0.08*</td>
<td>1.90 ± 0.10*</td>
</tr>
<tr>
<td>PCNA_{cap}/mm^2</td>
<td>5.4 ± 1.0</td>
<td>24.3 ± 5.8*</td>
<td>21.8 ± 4.5*</td>
<td>19.9 ± 4.4*</td>
<td>11.9 ± 4.3</td>
</tr>
<tr>
<td>PCNA_{int}/mm^2</td>
<td>27.7 ± 7.5</td>
<td>23.7 ± 8.9</td>
<td>23.5 ± 3.2</td>
<td>20.4 ± 2.3</td>
<td>13.3 ± 2.6</td>
</tr>
<tr>
<td>VEGF_{cap}/mm^2</td>
<td>92.0 ± 13.6</td>
<td>198.2 ± 35.4*</td>
<td>247.0 ± 18.9*</td>
<td>203.6 ± 25.5*</td>
<td>82.1 ± 9.4</td>
</tr>
<tr>
<td>VEGF_{noncap}/mm^2</td>
<td>137.5 ± 26.8</td>
<td>226.8 ± 19.4*</td>
<td>272.6 ± 27.0†</td>
<td>164.3 ± 5.1</td>
<td>89.3 ± 24.7</td>
</tr>
<tr>
<td>Percentage capillaries VEGF-positive</td>
<td>13.0 ± 2.4</td>
<td>27.5 ± 5.1*</td>
<td>29.4 ± 2.3*</td>
<td>22.3 ± 3.0</td>
<td>10.5 ± 2.1</td>
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|                |         |           |           |           |           |
| **Stimulated** |         |           |           |           |           |
| C:F            | 1.29 ± 0.04 | 1.43 ± 0.09 | 1.43 ± 0.06 | 1.67 ± 0.05* | 2.07 ± 0.12† |
| PCNA_{cap}/mm^2| 5.1 ± 3.4  | 107.8 ± 4.4* | 65.5 ± 20.0* | 61.6 ± 7.7* | 44.0 ± 4.2*  |
| PCNA_{int}/mm^2| 10.3 ± 1.3 | 46.2 ± 2.2* | 46.6 ± 19.5* | 60.4 ± 5.1* | 14.0 ± 3.4  |
| VEGF_{cap}/mm^2| 101.2 ± 7.5 | 119.8 ± 14.2 | 317.6 ± 18.2† | 395.1 ± 26.4† | —         |
| VEGF_{noncap}/mm^2| 151.9 ± 5.7 | 135.8 ± 12.3 | 338.9 ± 49.0* | 418.5 ± 17.5* | —         |
| Percentage capillaries VEGF-positive | 14.4 ± 1.0 | 15.4 ± 1.0 | 40.5 ± 3.7* | 41.2 ± 5.4* | 36.9 ± 3.2*  |

Note: C:F is capillary per fiber ratio. (⁎) p < 0.05 versus control; (†) p < 0.0001 versus control. Data for PCNA density in stimulated muscle from Haas and colleagues (30).
Table 1 shows that the density of VEGF-positive capillaries was similar in both control groups. There was a significant increase in VEGF$_{cap}$/mm$^2$ with prazosin treatment after 2 days, which was maintained up to 7 days and returned to control by 14 days (Fig. 3). With stimulation, there was no change in VEGF$_{cap}$/mm$^2$ after 2 days, but a considerable increase after 4 and 7 days, when it was greater than with prazosin (Fig. 3). The density of other VEGF-positive sites (interstitial and muscle fiber) was also increased earlier with prazosin, after 2 and 4 days, declining to control values later, whereas it increased later with stimulation (4 days) and continued to increase (Table 1). The proportion of capillaries

Figure 3. Photomicrographs of sections from EDL muscles stained by antibody to VEGF-A, which shows as brown reaction product, as follows: (A) control muscle with few positive sites; (B) negative control section after adsorption of antibody with VEGF peptide; (C) 2-day prazosin-treated muscle showing an increase in positive sites; (D) 14-day prazosin-treated muscle with few VEGF-positive sites, as in controls; (E) 4-day stimulated muscle with numerous VEGF sites; and (F) 14-day stimulated muscle also with plentiful positive sites. Bar represents 20 μm.
present that were positive for VEGF followed a similar trend to that of VEGF$_{cap}$/mm$^2$ (i.e., early increase after 2 and 4 days with prazosin with return to control values after 14 days), but a later increase with stimulation, after 4 days, that was maintained. The difference in time course of cell proliferation and VEGF expression between the two treatments becomes more obvious when values are expressed relative to controls. Figure 4 illustrates the relatively modest transient increase in capillary proliferation during prazosin treatment without proliferation of interstitial cells but accompanied by a substantial increase in the proportion of capillaries expressing VEGF. Stimulation, on the other hand, resulted in a striking increase in proliferation after 2 days, whereas VEGF expression did not increase until 4 days. Significant proliferation was maintained along with further increases in VEGF expression throughout the whole period of stimulation.

**Capillary Shear Stress**

Previous data demonstrated higher capillary shear stress after long-term prazosin treatment (5 weeks) or stimulation that increased capillary supply to a similar extent (18). To establish whether shear stress, capillary proliferation, and VEGF expression were associated during the course of angiogenesis, capillary V$_{rbc}$ and diameters were measured in resting EDL muscles at a time that VEGF expression was similar with both treatments (i.e., after 3 to 4 days). Capillary diameters were similar in stimulated (4.4 ± 0.2 mμm), prazosin-treated (4.6 ± 0.4 mμm), and control (4.4 ± 0.1 mμm) muscles, while V$_{rbc}$ was higher with both treatments (stimulation, 158 ± 7 mμm/s, $p < 0.05$; prazosin, 434 ± 127 mμm/s, $p < 0.01$) than in control muscles (100 ± 2 mμm/s). As the velocity was so much higher with prazosin (4-fold) than with stimulation (1.6-fold increase), calculated capillary shear stress was 4 times greater than control with prazosin but only 57% greater in stimulated muscles (Fig. 5).

**DISCUSSION**

This study shows that in skeletal muscles, where angiogenesis is induced by a long-term increase in blood flow, there is enhanced expression of VEGF protein at capillary locations prior to the appearance of increased capillary numbers. Capillary shear stress, even though estimated several hours after the last treatment had been applied, was much higher with prazosin than with stimulation and was associated with earlier VEGF upregulation. The time course of VEGF expression in capillaries was also different from that of their proliferation, as they increased concurrently with prazosin, but higher VEGF expression occurred after proliferation in stimulated muscles. Furthermore, prazosin did not lead to any change in proliferation of noncapillary...
interstitial nuclei, whereas stimulation led to a rapid, albeit transient, increase in proliferation of this cell type. These findings raise several important questions; namely, in which type of cell is VEGF upregulated, what is the stimulus for its upregulation, and what stimuli cause capillaries to proliferate before increases in VEGF expression in stimulated muscles.

Localization of VEGF in Skeletal Muscle

Although VEGF is considered to be the most important growth factor involved in angiogenesis during development (e.g., Ref. 12), tumor growth (7), and retinal neovascularization (1), and VEGF mRNA and protein are present in endothelial cells in development (45) and tumors (41), its localization in skeletal muscle has been studied to a limited extent. Increased levels of VEGF mRNA were found in contracting (9,25) and chronically stimulated muscles (31,47), but these studies did not identify the spatial expression of VEGF in relation to capillaries. Annex and colleagues (4) demonstrated VEGF immunostaining in some capillaries, but their illustrations also show staining of the matrix between muscle fibers. The current study shows both VEGF mRNA (Fig. 2) and protein (Fig. 3) occasionally appearing in the sarcolemmal region of muscle fibers, which would contribute to the increased density of noncapillary VEGF-positive loci in the interstitium, because it is not possible to distinguish between muscle and interstitial locations at the magnification used for counting. At capillary locations, VEGF-positive staining could be within or bound to (41) capillary endothelial cells themselves, or in other perivascular cell types such as pericytes, fibroblasts, or macrophages, all of which have been shown to express VEGF (7,48,49). We have previously shown, by electron microscopy, that pericyte coverage of capillaries is lower than normal in stimulated muscles, whereas there is very little change with prazosin treatment (21). The current finding of an increased proportion of capillaries positive for VEGF is therefore unlikely to be due to increased pericyte numbers. Macrophage accumulation was associated with an increase in capillarity in skeletal muscles developing collateral circulation after femoral artery occlusion (5). However, they were not observed previously in stimulated muscles (39) and there was no increase in the density of ED1-positive cells either before (2 days) or at the same time as (4 days, data not included) the density of noncapillary VEGF-staining increased. Fibroblasts, however, have been found using the electron microscope, lying adjacent to capillaries in stimulated muscles (22), and fibroblast-like elongated cells were observed in this study (see Fig. 2) and may therefore have contributed to the increased number of VEGF-positive cells, both capillary and noncapillary.

Possible Stimuli for Upregulation of VEGF

With respect to shear stress as a stimulus for upregulation of VEGF expression, this has seldom been studied either in vitro or in adult tissues, although there are several reports of activation of other growth factors by shear stress in vitro (see Ref. 14). A recent study by Gan and colleagues (24) reported alterations in VEGF expression in endothelial cells, by RT-PCR and by immunostaining, in human umbilical veins exposed to high laminar shear stress and constant pressure, whereby expression decreased, normalized, then decreased again over a 6-hour period of exposure. Because these are large conduit vessels and were perfused by buffer rather than blood for a relatively short period of time, the experimental conditions are very different from those of the current study where VEGF was studied in relation to capillaries in vivo over several days. The only other indication of the importance of flow and/or shear stress for expression of VEGF and the VEGF-R2 receptor is their strong presence in the endocardial endothelium in the outflow tract of the ventricle in the developing murine heart (38). There have been no data on expression of either VEGF mRNA or protein in any form of angiogenesis resulting from increased blood flow. Benoit and colleagues (6) reported increased VEGF mRNA expression in muscles of rats infused for 1 hour with sodium nitroprusside or acetylcholine, but as blood flow was restricted to resting levels by an arterial ligature, this does not support the hypothesis that increased blood flow alters VEGF expression. Moreover, their study

![Calculated capillary shear stress](image-url)
assessed changes in mRNA expression in the whole tissue, which does not permit conclusions about the role of blood flow on VEGF mRNA expression in vessels to be drawn.

Our hypothesis was that VEGF expression and proliferation would be increased via an elevation in capillary shear stress resulting from the increased blood flow, as both treatments used in this study increased blood flow acutely and chronically. Acute muscle contractions at 10 Hz increased blood flow ~15-fold (32), whereas during chronic stimulation, resting blood flow was higher in muscles stimulated for 2 days than in controls, and flow during contractions was higher in some, but not all, muscles stimulated for 3 days, and in all muscles stimulated for 7 days (20). Acute prazosin administration, at a dose corresponding to that consumed by rats drinking prazosin, trebled muscle blood flow (52), and chronic treatment for 14 days increased flow during contractions and decreased blood pressure (23), indicating that its efficacy was maintained. In the current study, mean arterial pressure with prazosin treatment was not different from controls (113 ± 6 mm Hg) after 2 and 4 days (108 ± 11 and 107 ± 11 mm Hg, respectively), but lower (90 ± 13 mm Hg) after 7 days, showing the progressive reduction of peripheral resistance. These data confirm that both treatments enhance blood flow, and hence shear stress, to skeletal muscle during the period of study. In addition, prazosin may have effects on the mechanical transduction of shear stress via its action of lowering plasma cholesterol (26), as cholesterol has been shown to modulate shear-induced activation of intracellular signaling pathways in vitro (28) and in endothelial culture (40).

Shear stress was estimated in capillaries under resting conditions at the time corresponding to a similar increase in proportion of capillaries expressing VEGF for the two treatments. Its calculation was based on measured capillary Vrbc and diameters, using viscosity from Sarelius’s (44) data. As there was no difference in capillary diameters between the two treatments and controls, and no correlation between diameters and Vrbc (r² = 0.032), shear stress was directly related to Vrbc (r² = 0.945), a parameter that can be measured with great accuracy. Shear stress was found to be considerably higher in prazosin-treated than stimulated muscles, which could be because the stimulus—increased blood flow—maintained through continuous consumption of the drug. Acute muscle contractions at 10 Hz were found to increase capillary shear stress by 35 ± 11% (15), but this could only be during the 8-hour stimulation period, while the current 57% increase was estimated during the 16-hour rest period after a few days’ chronic stimulation. On aggregate, a higher shear stress in capillaries during contractions, and throughout the rest period after 3 days (current study) or 7 days (18), indicates its possible importance for the elevation of VEGF expression in stimulated muscles.

Nevertheless, other factors, such as hypoxia or acidosis, both of which occur in skeletal muscles during heavy exercise, could also be involved in the upregulation of VEGF. Milkiewicz and colleagues (37) reported lower values of muscle PO2, measured within 20 minutes of termination of chronic stimulation for 2 days, in conjunction with an increased proportion of capillaries expressing VEGF protein. However, there were no signs of hypoxia in muscles stimulated for longer than 2 days (33), yet endothelial cell proliferation was still high after 14 days stimulation compared to control muscles, and an elevated proportion of VEGF-positive capillaries was maintained. Based on observations that VEGF expression can be increased in the heart (36,50), mesangial cells (27), and retinal pigment epithelial cells (46) by mechanical stretch and stress, or in bovine aortic endothelial cells by mechanical injury (13), another possible stimulus for VEGF expression in stimulated muscles could be the mechanical action of repeated contractions. This would provide a long-term stretch and relaxation of the sarcomeres and hence the capillaries, which are tethered to muscle fibers. Despite this being a repetitive stimulus throughout the period of stimulation, VEGF expression was increased to a similar extent by 3 or 56 days of stimulation (4), while capillarization increases gradually.

VEGF Expression and Capillary Proliferation

Capillary proliferation was estimated on the basis of PCNA immunostaining colocalized with capillaries by examination of adjacent cryostat sections. It was not possible to colocalize expression of VEGF to sites of nuclear proliferation because the intervening section was used for alkaline phosophatase staining, and the distance between VEGF and PCNA nuclear labeling was too great (~12 μm) to be certain of spatial congruity. Neither could the types of cell constituting the capillaries that were stained for PCNA (i.e., endothelial cells, pericytes, or adjacent fibroblasts) be ascertained. However, on the basis of our previous electron microscopy, it is unlikely that increased PCNA labeling would be due to proliferation of pericytes for the reasons outlined above. On the other hand, immuno-gold staining for BrdU showed...
equivalent proportions of labeled fibroblast and endothelial nuclei in muscles stimulated for 2 days, whereas only endothelial cells were labeled after 7 days (22). Thus, the large increase in PCNA labeling seen after 2 days of stimulation will include proliferating fibroblasts, whereas this is unlikely with longer durations.

Capillary-linked proliferation was greater in stimulated than prazosin-treated muscles, and while it coincided with the increased proportion of VEGF-positive capillaries in prazosin-treated animals, it was much greater and preceded VEGF upregulation in stimulated muscles. After 14 days, it returned to control values with prazosin treatment but was still several times higher than controls with stimulation. This temporal dissociation between the presence of such a potent angiogenic factor as VEGF and capillary-associated PCNA labeling in two models in which C:F ratio is increased to a similar extent by 14 days of either treatment, indicate that the mechanisms whereby capillaries increase in number are obviously different. This supports our previous data showing stimulus-dependent forms of angiogenesis; that is, capillary growth by proliferation and sprouting with stimulation, and capillary splitting with prazosin treatment (22). Furthermore, whereas with prazosin it is possible that this proliferation may be associated with the observed increase in shear stress, which is known to stimulate endothelial cell growth in vitro (3), the far greater proliferation in stimulated muscles is likely due to additional factors. Other studies have suggested a role for FGF-2 in this model, but this was found only in larger vessels, not capillaries, in stimulated muscles (11), and it was not upregulated in acutely contracting muscles (9,43). On the other hand, Brown and colleagues (10) have reported elevation of a small-molecular-weight endothelial cell stimulating angiogenic factor (ESAF) in stimulated muscles which correlated with increased capillary supply. Another likely factor in capillary proliferation is nitric oxide (NO), which is known to be released by shear stress and appears to have a cooperative relationship with VEGF in angiogenesis. Inhibition of nitric oxide synthase attenuated upregulation of VEGF mRNA by exercise (25), and we have shown that increased C:F ratio and capillary labeling index for bromodeoxyuridine were inhibited in stimulated muscles by concurrent nitric oxide synthase inhibition (35). While there is evidence that the proangiogenic effect of VEGF acts via NO (53), we speculate that a synergistic effect may exist, whereby shear stress could induce VEGF expression in parallel with NO.

In conclusion, we have demonstrated increased VEGF expression at capillary sites in skeletal muscles where blood flow was increased chronically by vasodilator treatment or electrically induced contractions. Our results indicate a strong association between shear stress and capillary VEGF expression, particularly in prazosin-treated animals where the primary stimulus is increased blood flow, and are consistent with the view that mechanical factors play an important role in the regulation of capillary growth, although they do not allow conclusions about a causal relationship between these two parameters. Furthermore, differing patterns of cell proliferation in the two models of hyperemia-induced capillary growth suggest that angiogenesis was induced by more than one factor.

REFERENCES


