Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis

IRINA RIVILIS,1 MALGORZATA MILKIEWICZ,2 PAMELA BOYD,1 JASON GOLDSTEIN,1 MARGARET D. BROWN,2 STUART EGGINTON,2 FAY M. HANSEN,3 OLGA HUDLICKA,2 AND TARA L. HAAS1
1Department of Kinesiology and Health Sciences, York University, Toronto, Ontario, Canada M3J 1P3; 2Department of Physiology, University of Birmingham, Birmingham, United Kingdom B15 2TT; and 3Department of Biological Sciences, Oakland University, Rochester, Michigan 48309-4401

Capillary growth in skeletal muscle occurs via the dissimilar processes of abluminal sprouting or longitudinal splitting, which can be initiated by muscle stretch and elevated shear stress, respectively. The distinct morphological hallmarks of these types of capillary growth suggest that discrete sets of angiogenic mediators play a role in each situation. Because proteolysis and proliferation are two key steps associated with capillary growth, we tested whether differences in the regulation of matrix metalloproteinases (MMPs) or VEGF may be associated with the two types of capillary growth. We found significant increases in MMP-2 total protein and percent activation, and membrane type-1 MMP mRNA levels, compared with controls after muscle stretch but not after shear stress stimulation. In contrast, VEGF protein and endothelial cell proliferation increased after either angiogenic stimulus. We observed that MMP-2 regulation occurs independent of VEGF signaling, because VEGF did not induce MMP-2 production or activation in isolated endothelial cells. Our data suggest that the involvement of MMPs in capillary growth is dependent on the nature of the angiogenic stimulus.

matrix metalloproteinase

ANGIOGENESIS, or capillary growth, occurs in both cardiac and skeletal muscle in response to mechanical and humoral stimuli present during exercise (24). Elevated shear stress and muscle stretch each induces capillary growth in skeletal muscle independently of an exercise stimulus (25). Prolonged elevation of capillary shear stress can be accomplished through chronic administration of vasodilators (55), such as the α1-adrenergic receptor antagonist prazosin (15, 16, 36), causing significant increases in capillary number after 1 wk of treatment (53). Similarly, overloading the extensor digitorum longus (EDL) muscle, which results in a sustained 20% increase in sarcomere length with minimal alterations in blood flow (17), induces capillary growth before muscle hypertrophy, with significant increases in capillary number after 1 wk and greater increases after 2 wk (52).

Interestingly, the process of capillary formation differs significantly dependent on whether the microcirculation is stimulated by increased shear stress or by muscle stretch. Capillary growth is described conventionally to begin with endothelial cell activation, which initiates proteolysis of the basement membrane, enabling abluminal sprout formation, cell proliferation, and subsequent growth of a new capillary (2, 41). This process is the predominant mechanism of capillary growth in muscles activated by chronic electrical stimulation or in stretched muscle, with the exception that endothelial cell proliferation precedes proteolysis and sprout formation (18, 23). However, shear stress stimulation causes capillary growth via longitudinal splitting of the capillary and not by abluminal sprouting (18, 53). During longitudinal splitting, activated capillary endothelial cells form sprouts that grow into the lumen, eventually dividing the capillary into two distinct lumena. This process may not involve proteolysis, because the capillary restructures luminally without an apparent requirement for basement membrane dissolution. Electron microscopic studies have failed to note focal disappearance of basement membrane subsequent to shear stress stimulation as is observable during chronic electrical stimulation or muscle stretching (52, 53).

Thus two mechanisms of capillary growth can be initiated dependent on the nature of the initial angiogenic stimulus, implying that the molecular events driving the process of capillary growth differ dependent on the stimulus. Cell proliferation and basement membrane proteolysis are both considered to be essen-
tial for the process of capillary growth. Cell proliferation likely is mediated by VEGF, a potent endothelial cell mitogen (42, 50). Capillary growth in chronically stimulated skeletal muscle was inhibited by concurrent infusion of antibodies against VEGF (1). Degradation of the capillary basement membrane is accomplished by a family of zinc- and calcium-dependent enzymes known as matrix metalloproteinases (MMPs) (7). MMPs play a major role in the process of tumor angiogenesis (27, 37, 47). Recently, we demonstrated the importance of MMPs in a model of activity-induced angiogenesis through prevention of basement membrane degradation and abluminal capillary sprouting by inhibiting MMP activity (23).

Comparison of shear stress and muscle stretch models of skeletal muscle angiogenesis provides us with the unique opportunity to test whether the distinct angiogenic stimuli that cause disparate patterns of capillary growth can be associated with differential production of key angiogenic mediators, such as VEGF and MMPs.

In the current study, we tested the hypothesis that MMP-2 is upregulated by stimuli that elicit abluminal sprouting, such as muscle stretch, and not by stimuli that cause longitudinal capillary splitting, such as increased shear stress. However, we considered that both types of capillary growth would involve increased production of VEGF.

METHODS

Animal protocols. Muscle stretch of adult male Sprague-Dawley rats was performed as previously described (52). Briefly, the EDL muscle was overloaded by unilateral extirpation of the tibialis anterior under inhalation anesthesia by 2% halothane (Fluothane, ICI). This procedure results in a facilitation of the tibialis anterior under inhalation anesthesia by flurotamine (gift of Pfizer; Sandwich, UK) in their drinking water performed in accordance with the United Kingdom Animals

nosal anesthesia (50 mg/kg ip). All animal protocols were controls. EDL muscles were removed under pentobarbitone

ment and 2-fold at 5 wk posttreatment) (15, 36). Additionally, calculated capillary shear stress (8) was increased from 48 h (muscle samples) or 24 h (endothelial cells) at 37°C in buffer composed of 5 mM CaCl₂ and 50 mM Tris·HCl (pH 8.0) before staining with Coomassie brilliant blue. Gels were destained until the desired resolution was obtained, digitzed, and then analyzed using the Fluorchem system (AlphaInnotech).

mRNA isolation and Northern blot analysis. Muscle samples were powdered and then homogenized in TRIzol (GIBCO-BRL), and total cellular RNA was isolated according to the manufacturer’s directions. Total RNA (20 μg) from each sample was separated on a 1.2% agarose-formaldehyde gel. Gels were stained with Sybr green (Sigma) to assess RNA integrity and lane loading and then transferred overnight onto a nylon membrane (GeneScreen Plus, NEN Life Science Products). After transfer, RNA was UV cross-linked to the membrane. The blots were prehybridized for 1 h, then hybridized with [α-32P]dTTP dDNA probes specific for membrane type-1 (MT1)-MMP (22), prepared using random primer DNA labeling (Roche), and spin purified (G50 columns; Pharmacia). Blots were washed with 2× SSC at room temperature, followed by 2× SSC with 2% SDS at 65°C to remove nonspecific binding, and then exposed to Hyperfilm MP film (Amersham Pharmacia Biotech) for 2–5 days at 80°C. Autoradiographs were digitized and then quantified by densitometry using the Fluorchem gel doc system. Band intensities were normalized to 28S rRNA intensity to correct for loading differences. Intensities were then expressed relative to the intensity of the first control sample loaded on the gel.

Immunohistochemistry. Frozen sections (6 μm) of EDL muscle from stretch- or shear stress-stimulated rats were fixed in cold acetone for 5 min and then incubated in 3% hydrogen peroxide in methanol and 10% normal horse serum to block endogenous peroxidase activity and nonspecific antibody binding. They were incubated with mouse monoclonal antibodies against MMP-2 (R&D) or CD31 [platelet-endothelial cell adhesion molecule (PECAM-1); BD Bioscience] at 4°C

Madri and Williams (34). Cells were grown on gelatin-coated tissue culture plates (1.5% gelatin in phosphate-buffered saline) and maintained in DMEM (Sigma) containing 25% sterile-filtered conditioned bovine aortic endothelial cell medium, 10% fetal bovine serum, 50 units penicillin, 0.05 mg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine. For experiments, 500,000 cells were plated per 35-mm dish (precoated with type I collagen at 12 μg/ml). Recombinant human VEGF (Calbiochem) was administered at doses ranging from 10 to 100 ng/ml in media containing 10% fetal bovine serum without conditioned medium. After 24 h, media were replaced with fresh VEGF-containing media. After 48 h, cells were trypsinized and counted by hemocytometer or were lysed (120 mM Tris·HCl, 0.01% Triton X-100, and 5% glycerol; no protease inhibitor), and protein was quantified by BCA assay (Pierce Biochemicals).

Protein isolation. Frozen muscles were powdered, and total cellular protein was isolated by homogenization of the muscles samples in lysis buffer (120 mM Tris·HCl and 5% glycerol; no protease inhibitor). After homogenization, Triton X-100 was added to a final concentration of 0.1% (23). Protein concentrations of lysates were calculated using a BCA assay.

Zymography. Gelatin zymography was performed on protein lysates from rat muscles or VEGF-treated endothelial cells to analyze total MMP-2 protein and the ratio of latent to active forms. Fifteen micrograms of muscle samples or 10 μg of cell culture lysates were size fractionated under nonreducing conditions through a 10% SDS-polyacrylamide gel impregnated with 0.04% gelatin (22). Gels were incubated for 48 h (muscle samples) or 24 h (endothelial cells) at 37°C in buffer composed of 5 mM CaCl₂ and 50 mM Tris·HCl (pH 8.0) before staining with Coomassie brilliant blue. Gels were destained until the desired resolution was obtained, digitzed, and then analyzed using the Fluorchem system (AlphaInnotech).
Table 1. Capillary-to-fiber ratio values for EDL muscles from stretch or shear stress-stimulated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 Days</th>
<th>4 Days</th>
<th>7 Days</th>
<th>14 Days</th>
<th>28 Days</th>
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<td>Shear stress</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>
<td>ND</td>
</tr>
<tr>
<td>Muscle stretch</td>
<td>1.22 ± 0.05</td>
<td>ND</td>
<td>1.20 ± 0.05</td>
<td>1.32 ± 0.12</td>
<td>1.68 ± 0.13*</td>
<td>2.07 ± 0.10*</td>
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Values are means ± SE. EDL, extensor digitorum longus; ND, not determined for this time point. *P < 0.01 compared with control samples.

RESULTS

Prazosin administration, causing elevated shear stress within capillaries of EDL muscles, resulted in a significant increase in the capillary-to-fiber ratio within 7 days and a further increase at 14 days (Table 1). This is comparable with results from stretch-stimulated samples. Prazosin treatment (Fig. 1A) illustrates MMP-2 levels in control and drug-treated animals. A faint band corresponding to active MMP-2 (62 kDa) appeared in most samples. Figure 1B illustrates MMP-2 levels in control and stretch-stimulated muscle samples. Both latent and active forms of MMP-2 were clearly detectable after muscle stretch. Note that zymographic activity corresponding to other MMPs, namely MMP-9 (92 kDa gelatinase), was absent from both prazosin-treated and stretch-stimulated muscle samples.

Densitometric analyses of the zymography gels are summarized in Fig. 2. Both the total amount of MMP-2 present and the percentage of MMP-2 in the active form were calculated for shear stress- and muscle stretch-stimulated samples. Prazosin treatment (Fig. 2A) did not significantly affect the levels of total MMP-2 protein at any time point. The percentage of MMP-2 present in the 62-kDa active form did not significantly increase and, in fact, showed a significant decrease at 14 days compared with controls. Conversely, stretch treatment of the EDL muscle (Fig. 2B) resulted in a significant increase in the total amount of MMP-2 protein compared with controls at day 4 (P = 0.03) and a trend to remain elevated at days 7 and 14 (P = 0.07 compared with control), with levels returning to control by day 28. Furthermore, the percentage of active MMP-2 was approximately fourfold greater than controls at the 4-day time point and showed a nonsignificant trend to remain elevated at days 7 and 14, with a return to control value by 28 days. Thus muscle stretch appears to be a much more potent stimulus for the increase of both total MMP-2 and active MMP-2 than shear stress stimulation.

Consistent with zymography findings, immunostaining of MMP-2 in muscle sections from control (Fig. 3A) or prazosin-treated (Fig. 3E) animals showed minimal positive staining, whereas stretch-stimulated muscles showed obvious increases in MMP-2 staining intensity (Fig. 3C). By examination of adjacent sections immunostained for endothelial cell-specific PECAM-1 (Fig. 3, B, D, and F), it appears that MMP-2 is localized to capillary structures rather than to interstitial or skeletal muscle cells.

Previous studies have demonstrated a coincident regulation of expression of MMP-2 and MT1-MMP in cultured endothelial cells and in vivo (22, 23). In fact, effective processing of MMP-2 to the active form requires the presence and activity of MT1-MMP (38, 54). Thus zymography analysis of stretch-stimulated muscles, which showed significant increases in the active
form of MMP-2 compared with control muscles, provides indirect evidence of increased production of MT1-MMP as a result of muscle stretch. We verified that MT1-MMP is upregulated during muscle stretch by Northern blotting (Fig. 4). MT1-MMP mRNA significantly increased in stretch-stimulated (2.17 ± 0.25 at day 7 compared with 1.22 ± 0.19 in controls), but no effect was seen on MT1-MMP mRNA levels during prazosin treatment of muscles. As expected from zymography results, MMP-2 mRNA levels also showed a trend to increase during stretch (7-day time point) but not during shear stress stimulation (Fig. 4). Unfortunately, the 4-day stretch samples were not analyzable due to RNA degradation.

VEGF is a known stimulator of endothelial cell proliferation (6, 31, 39). Endothelial cell proliferation occurs in prazosin-treated skeletal muscle coincident with increased levels of VEGF protein (36), and, in the current study, we documented this occurrence in stretch-stimulated skeletal muscle. We found significant but transient increases in VEGF protein associated with capillaries as well as interstitial and skeletal

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**Fig. 2.** Analysis of total MMP-2 and percent active MMP-2 in stimulated muscles. Intensity measurements from zymographic bands were utilized to compare total MMP-2 protein (total of latent and active band intensities) and percent active MMP-2 (ratio of active to latent band intensity) for prazosin-treated muscles (A) and stretch-stimulated muscles (B). The left axis and bar graph represent the total MMP-2 protein (normalized to control, which is set to an intensity of 1). The right axis and line graph represent the percent active MMP-2 at each time point. *Significantly different from control (P < 0.05); + significantly different from all other time points (P = 0.01); # values with a trend to significance (P = 0.07).

**Fig. 3.** Immunostaining of prazosin-treated and stretch-stimulated muscles. Consecutive sections of extensor digitum longus (EDL) muscle were stained for MMP-2 (A, C, and E) or CD31 (platelet-endothelial cell adhesion molecule-1; B, D, and F) to define capillary locations. A and B: control (nonstimulated) muscles; C and D: stretch-stimulated muscles at the 4-day time point; E and F: prazosin-treated muscles at the 4-day time point. Arrows in MMP-2-stained samples point to MMP-2-positive capillaries. Arrows in CD-31-stained samples point to capillary locations. All sections were counterstained with hemotoxylin to define locations of nuclei.
Fig. 4. Membrane type-1 (MT1)-MMP and MMP-2 mRNA increase in response to stretch but not prazosin stimulation. Representative Northern blots (A) and their respective quantification (B) are provided for prazosin and stretch stimulation. Band intensities were first normalized to the intensity of 28S rRNA to correct for loading variations and then compared with control sample intensities. Open bars represent MT1-MMP and solid bars represent MMP-2 mRNA values. Control values deviate slightly from 1 due to loading of multiple control samples on the same gel. n = 3–5 muscles analyzed for each time point. *Significantly different from control (P < 0.05); #values with a trend for significance from control (P = 0.06); + significantly different from the 7-day time point (P < 0.05).

DISCUSSION

This study provides evidence that stimulus-specific production of angiogenic mediators may contribute to the significant morphological differences in capillary growth resulting from shear stress compared with muscle stretch stimulation. We detected increased production of MMP-2 and MT1-MMP during muscle stretch- but not during prazosin-induced capillary growth. On the other hand, increased production of VEGF and capillary-associated proliferation occurred in both models of capillary growth.

Active MMPs contribute to extracellular matrix proteolysis and increased cell invasiveness in many cell types including endothelial cells (11, 46, 47). Specifically, MMPs are considered to be necessary for the process of abluminal capillary sprouting because of their critical roles in basement membrane and interstitial matrix degradation (41, 14). Inhibition of MMP activity disrupts angiogenesis in a variety of experimental systems (37, 44, 45), including the exercise-induced increase in capillary number that accompanies chronic stimulation of skeletal muscle (23). Previously, we demonstrated upregulation of MMP-2 and MT1-MMP during chronic electrical stimulation of rat skeletal muscle (23). These two MMPs act synergistically to degrade basement membrane matrix molecules (38).

Table 2. Quantification of PCNA and VEGF staining of muscle stretch samples

<table>
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<th>Control</th>
<th>4 Days</th>
<th>7 Days</th>
<th>14 Days</th>
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<tr>
<td>PCNA_{cap/mm^2}</td>
<td>9.0 ± 3.00(3)</td>
<td>20.19 ± 6.35(4)</td>
<td>64.56 ± 3.64*(6)</td>
<td>22.84 ± 2.68*(8)</td>
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<tr>
<td>PCNA_{int/mm^2}</td>
<td>6.0 ± 0.58(3)</td>
<td>46.15 ± 3.14*(4)</td>
<td>64.86 ± 8.5*(6)</td>
<td>19.61 ± 5.33*(8)</td>
</tr>
<tr>
<td>VEGF_{cap/mm^2}</td>
<td>90.80 ± 8.04(3)</td>
<td>170.19 ± 13.54*(4)</td>
<td>175.86 ± 21.53*(3)</td>
<td>134.48 ± 11.09(3)</td>
</tr>
<tr>
<td>%Capillaries</td>
<td>11.42 ± 1.10(3)</td>
<td>17.14 ± 1.14(4)</td>
<td>27.30 ± 5.00*(3)</td>
<td>13.79 ± 1.86(3)</td>
</tr>
<tr>
<td>VEGF_{noncap/mm^2}</td>
<td>158.62 ± 17.70(3)</td>
<td>279.80 ± 48.71(4)</td>
<td>270.12 ± 46.02(3)</td>
<td>178.16 ± 20.72(3)</td>
</tr>
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Values are means ± SE; numbers in parentheses indicate numbers of samples. PCNA_{cap}, and VEGF_{cap}, PCNA and VEGF associated with capillaries; PCNA_{int}, PCNA associated with interstitial cells; VEGF_{noncap}, VEGF in noncapillary-associated cells. *Significantly different from controls (P < 0.05).
In the current study, we detected increased production and activation of MMP-2, as well as production of MT1-MMP, during stretch-induced angiogenesis. Immunostaining showed MMP-2 present at capillaries, compatible with endothelial cell localization. Similar to the patterns of expression seen during chronic electrical stimulation, the muscle stretch induced increases in MMP-2 and MT1-MMP mRNA, and MMP-2 protein and activity levels were transient, and preceded detection of significant increases in capillary numbers. The enhanced production of MMP-2 and MT1-MMP is consistent with the observation that stretch-induced capillary growth occurs via the process of abluminal sprouting and that this process requires regulated basement membrane dissolution via MMP activity.

The specific mechanism by which muscle stretch induces MMP-2 and MT1-MMP production is not yet known. Stretch may act directly on the capillary endothelial cells, because endothelial cells are capable of detecting mechanical strain and responding through activation of kinase pathways and alterations in gene transcription (20, 26). Similarly, culture of microvascular endothelial cells within three-dimensional collagen elicits upregulation of MMP-2 and MT1-MMP that is thought to occur as a result of tensional forces that develop as the cells remodel their surrounding matrix (22). However, other cell types resident within the muscle tissue (skeletal myocytes, satellite cells, fibroblasts, pericytes) may also act as sensors of the stretch stimulus (33), producing MMPs themselves (35) or releasing factors that stimulate endothelial cell production of MMPs (3, 21).

On the other hand, prazosin treatment, through dilation of upstream arterioles, affects hemodynamic forces acting on the capillary luminal surface. It has been hypothesized that prazosin may increase capillary circumferential wall tension (43), which varies directly as a function of luminal pressure and vessel radius. Calculations of capillary circumferential wall tension have not been made because of difficulties in accurately measuring capillary pressures. However, because capillaries distend with increased luminal pressure (30) and lack the ability to actively control their diameter, any increase in luminal pressure should be detectable as an increase in capillary diameter. During prazosin treatment, capillary diameters remained constant (36) or were decreased slightly (16), suggesting that changes to capillary pressure, and thus circumferential wall tension, are minimal in this model despite vasodilation of upstream arterioles. Thus the primary effect of prazosin treatment appears to be the modulation of capillary shear stress (25), because calculated capillary shear stress increased fourfold after 4 days of prazosin treatment (36).

Despite evidence that shear stress activates multiple endothelial cell signaling pathways (12, 19, 4), we found no evidence for regulation of MMP-2 production or activity, or MT1-MMP production, during shear stress-stimulated capillary growth. Our results directly support previous ultrastructural studies that failed to note breakdown of the capillary basement

Fig. 5. VEGF protein is increased by both prazosin and stretch stimulation of EDL muscles. Representative Western blots and their quantification are shown for prazosin treatment (A) and stretch stimulation (B) of EDL muscles. VEGF protein levels were normalized to that of control muscles in each group; n = 3 or 4 muscles analyzed for each time point. *Significantly different from control (P < 0.05).

Fig. 6. Effect of VEGF on cultured microvascular endothelial cells. A: endothelial cell proliferation, measured as an increase in cell number, was observed for all doses of recombinant VEGF protein. In B, the effect of VEGF treatment on MMP-2 production and activity was assessed by zymography. Quantification of zymographic bands (C) shows no significant change in total MMP-2 protein or percent active MMP-2. n = 3 for all time points.
membrane during shear stress-induced angiogenesis (18, 53) and strongly suggest that the process of capillary growth stimulated by elevated shear stress occurs independent of MMP-driven proteolytic events.

Our inability to detect altered levels of MMP-2 or MT1-MMP with shear stress stimulation contrasts with the findings of studies that demonstrated enhanced MMP-2 production and activity in large arteries subjected to elevated shear stress (48), or to low flow and decreased shear stress (5). However, a third study using isolated coronary arteries found no sensitivity of MMP-2 production or activation to decreased or increased shear stress (13). Given the range of reported observations in these three studies, significant doubt remains as to whether MMP-2 production is sensitive to altered shear stress in either macrovessels or microvessels. Our observations lead to the conclusion that microvascular endothelial cells do not regulate MMP-2 production or activation in response to elevated shear stress.

A previous study found elevated levels of MMP-9 during prazosin stimulation of skeletal muscle (49). However, those authors found MMP-9 expression associated with microvessels of a diameter >20 μm rather than with capillaries, and they hypothesized a role for MMP-9 in the process of arteriolarization. We saw no evidence of MMP-9 expression by zymography in the samples we analyzed from either stretch- or prazosin-stimulated muscles and thus do not consider MMP-9 to be a significant factor in capillary growth in these muscles.

In contrast to MMP-2 and MT1-MMP, we observed significant but transient elevations in VEGF protein levels after either muscle stretch or prazosin stimulation. Our data suggest that VEGF may be critical for both abluminal sprouting and longitudinal splitting processes of capillary growth. The increased production of VEGF by capillary-associated cells and a trend to increased VEGF in interstitial and skeletal muscle cells after muscle stretch are consistent with previously published reports of cell stretch-induced VEGF production in cardiac myocytes and coronary endothelial cells (32, 51). It is possible that interstitial cells such as fibroblasts also directly respond to stretch by synthesizing VEGF. Enhanced proliferation, as detected by PCNA staining, of capillary-associated cells may be due to the direct mitogenic effects of VEGF, although the role of other factors and signaling pathways in stimulating proliferation cannot be disregarded at this time. On the other hand, proliferation of interstitial cells likely occurs independent of VEGF because these cells lack the receptors to respond to VEGF. It has been shown that stretch stimulates proliferation of fibroblasts, but the signaling pathways are as yet unknown (8, 28).

We showed by Western blotting that prazosin treatment upregulates expression of VEGF. This may occur through shear stress stimulated increases in endothelial cell nitric oxide production (10), because nitric oxide is known to stimulate transcription of VEGF in endothelial cells (9, 40). The capillary-associated proliferation documented by Milkiewicz and colleagues (36) is consistent with a proliferative role of VEGF (50) or directly of nitric oxide (56). The significant increase in nitric oxide and decreased shear stress versus abluminal muscle stretch results in two distinct patterns of cell signaling, gene activation, and endothelial cell behavior. At this point, little is known about the molecules involved in the detection of these angiogenic stimuli or the activated signaling pathways. We now know that the production of MMP-2 and MT1-MMP is sensitive to stretch activation but not to shear stress activation, and this correlates both with the occurrence of abluminal sprouting in stretched-stimulated muscles and with the absence of abluminal sprouting in shear-stress-stimulated muscles. However, production of VEGF can be triggered by either stimulus. Future work delineating the signaling pathways initiated by both types of angiogenic stimuli will shed light on the differential regulation of the MMPs and will help to identify other key gene products uniquely essential for each process of capillary formation.

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