Journal of Cellular Physiology

p38 MAPK Activity Is Stimulated by Vascular Endothelial Growth Factor Receptor 2 Activation and Is Essential for Shear Stress-Induced Angiogenesis

ERIC GEE,[1] MALGORZATA MILKIEWICZ,[1,2] AND TARA L. HAAS[1]*

[1] *School of Kinesiology and Health Science, Muscle Health Research Centre, York University, Toronto, Ontario, Canada*

[2] *Department of Laboratory Diagnostics and Molecular Medicine, Pomeranian Medical University, Szczecin, Poland*

Increased capillary shear stress induces angiogenesis in skeletal muscle, but the signaling mechanisms underlying this response are not known. We hypothesize that shear stress-dependent activation of vascular endothelial growth factor receptor 2 (VEGFR2) causes p38 and ERK1/2 phosphorylation, which contribute to shear stress-induced angiogenesis. Skeletal muscle microvascular endothelial cells were sheared (12 dynes/cm^2, 0.5–24 h). VEGFR2-Y1214 phosphorylation increased in response to elevated shear stress and VEGF stimulation. p38 and ERK1/2 phosphorylation increased at 2 h of shear stress but only p38 remained phosphorylated at 6 and 24 h of shear stress. VEGFR2 inhibition abrogated p38, but not ERK1/2 phosphorylation. VEGF production was increased in response to shear stress at 6 h, and this increased production was abolished by p38 inhibition. Male Sprague–Dawley rats were administered prazosin (50 mg/L drinking water, 1, 2, 4, or 7 days) to induce chronically elevated capillary shear stress in skeletal muscle. In some experiments, mini-osmotic pumps were used to dispense p38 inhibitor SB203580 or its inactive analog SB202474, to the extensor digitorum longus (EDL) of control and prazosin-treated rats. Immunostaining and Western blotting showed increases in p38 phosphorylation in capillaries from rats treated with prazosin for 2 days but returned to basal levels at 4 and 7 days. p38 inhibition abolished the increase in capillary to muscle fiber ratio seen after 7 days of prazosin treatment. Our data suggest that p38 activation is necessary for shear stress-dependent angiogenesis.

J. Cell. Physiol. 222: 120–126, 2010.

Shear stress regulates vascular development in the embryo and vascular tone, arteriogenesis, and angiogenesis in the adult (Resnick et al., 2003). This hemodynamic stimulus activates numerous mechanoreceptors on the endothelium such as integrins, G protein-coupled receptors, cell adhesion molecules, ion channels, and receptor tyrosine kinases (Chien, 2007). Shear stress also activates numerous signaling molecules such as protein kinase C, focal adhesion kinase, c-Src, Rho family GTPases, phosphoinositide 3-kinases, and mitogen-activated protein kinases (MAPKs) (Li et al., 2005). Transcription factors such as Ets-1 (Milkiewicz et al., 2008), NF-κB, Krüppel-like factor 2 (Chien, 2007), c-Myc, activator protein 1, and T-cell factor (Li et al., 2005) are known to be regulated by shear stress.

Both VEGFR2 and NO are implicated as key players in shear stress-dependent angiogenesis. Vascular endothelial growth factor receptor 2 (VEGFR2) is implicated as both a ligand-activated receptor (Lamalice et al., 2004) and a mechanoreceptor (Jin et al., 2003). Activation of VEGFR2 occurs immediately in response to elevated shear stress and is not prevented by the presence of a polyclonal anti-VEGF antibody, suggesting that activation is not induced by ligand binding (Chen et al., 1999). Shear stress activation of VEGFR2 recruits the adaptor molecules Shc, Grb2, and Sos (Chen et al., 1999). VEGFR2 is known to initiate multiple downstream signaling pathways including the activation of Ras/MEK/ERK, Src, PI3K/Akt, HSP90, focal adhesion kinase, and p38 (Traub and Berk, 1998; Le Boeuf et al., 2004; Jin et al., 2005). Shear stress increases endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production via activation of Akt (Jin et al., 2003), and induces phosphorylation of ERK1/2 and p38 MAPKs (Milkiewicz et al., 2006; Chien, 2007). Sequestration of VEGF or inhibition of nitric oxide synthase (NOS) activity abolishes shear stress-mediated luminal projection formation and angiogenesis (Baum et al., 2004; Williams et al., 2006).

Shear stress-dependent interaction of vascular endothelial (VE)-cadherin with VEGFR2 occurs within 1–2 min of shear stress, resulting in p38 phosphorylation (Shay-Salit et al., 2002). p38 has been demonstrated to be both anti- (Gratton et al., 2001) and pro-angiogenic (Yang et al., 2001; Rocic et al., 2007). Gratton et al. (2001) reported that p38 depresses Akt levels resulting in apoptotic signaling. Similarly, serum starvation induced apoptosis in neuronal cells that was mediated by p38 (Gomes and Rockwell, 2008). However, p38α deletion results in homozygous embryonic lethality due to reduced

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: Heart and Stroke Foundation;
Contract grant number: NA6089.
Contract grant sponsor: Canadian Institutes of Health Research;
Contract grant number: MOP 53272.
Contract grant sponsor: National Sciences and Engineering Research Council of Canada Postgraduate Scholarship.
Contract grant sponsor: Polish Ministry of Science and Higher Education;
Contract grant number: N N401 051833.

*Correspondence to: Tara L. Haas, School of Kinesiology and Health Sciences, Rm. 341 Farquharson, York University, 4700 Keele St., Toronto, ON, Canada M3J 1P3. E-mail: thaas@yorku.ca

Received 13 March 2009; Accepted 6 August 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 22 September 2009.
DOI: 10.1002/jcp.21924

vascularization and increased apoptosis (Mudgett et al., 2000), suggesting that p38 provides a positive stimulus to sustain the vasculature. VEGF-induced cell migration requires p38 (Kanno et al., 2000) and VEGF-induced VEGFR2-dependent p38 phosphorylation is necessary for in vitro tube formation (Yang et al., 2001; Rocic et al., 2007). In vivo, ischemia/reperfusion-induced coronary collateral growth is reduced by 50% following p38 inhibition (Rocic et al., 2007), again supporting a pro-angiogenic role for p38 activity.

Chronically elevated capillary shear stress causes a distinct form of angiogenic remodeling that has been referred to as luminal splitting (Zhou et al., 1998). During luminal splitting, the endothelial cells of the capillary form luminal protrusions that internally divide the single capillary (Zhou et al., 1998). These "luminal bridges" become permanent dividers as the single capillary remodels to become two parallel capillaries. The additional flow path may reduce shear stress through the capillary network and provide improved oxygen delivery. Luminal splitting occurs in the absence of significant endothelial cell proliferation or basement membrane degradation (Rivilis et al., 2002). However, cellular signals needed for luminal splitting are not well characterized. We hypothesize that shear stress-dependent activation of VEGFR2 increases the phosphorylation of ERK1/2 and p38, and that these MAPKs may contribute to the process of shear stress-mediated angiogenesis.

Methods

Cell Culture

Skeletal muscle microvascular endothelial cells were isolated from the extensor digitorum longus (EDL) of male Sprague–Dawley rats as previously described (Han et al., 2003). The cells were cultured in gelatin-coated flasks with growth media (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% Glutamax (Invitrogen, Burlington, ON), 0.11 mg/ml sodium pyruvate) at 7% CO_2 and 37°C for a maximum of 10 passages.

In Vitro Shear Stress

Cells were exposed to shear stress, as described by Milkiewicz et al. (2006) for 0.5, 2, 6, or 24 h. Briefly, cells were grown to confluence overnight on gelatin-coated glass cover slips and mounted in a laminar flow chamber. A dual head pump (Harvard Apparatus, St. Laurent, QE) connected to parallel laminar closed flow chambers (FCS2, Bioptechs, Butler, PA) was used to pump media generating shear stress of 12 dynes/cm^2. For assessment of VEGF secretion, Opti-MEM (Gibco, Burlington, ON), a reduced serum media, was pumped over the cells and collected. VEGFR2 was inhibited using 10 μM VEGFR2 kinase inhibitor I (VEGFR2i) (Calbiochem 676480, San Diego, CA). Efficacy of VEGFR2i inhibition of VEGFR2 (Y1214) phosphorylation was assessed in pre-treated cells followed by 5 min 20 ng/ml VEGF (Cedarlane Laboratories, Burlington, ON) stimulation by Western blot. ERK1/2 and p38 phosphorylation was measured after cells were stimulated with 20 ng/ml of VEGF for 30 min. To inhibit eNOS or p38, cells were treated with 30 μM Omega *N*-nitro-L-arginine (LNNA) (Sigma, Oakville, ON) (Milkiewicz et al., 2006) or 10 μM SB203580 (Tocris, Ellisville, MS), respectively. Cells were pretreated with the inhibitors for 1 h prior to shear stress exposure.

In Vivo Shear Stress

Male Sprague–Dawley rats (150–200 g) were administered prazosin (50 mg/L drinking water, ad libitum), an alpha$_1$-adrenergic receptor inhibitor, which is known to induce chronic vasodilation, and was reported previously to increase EDL blood flow threefold (Ziada et al., 1989), thus inducing a sustained capillary shear stress of approximately 15 dynes/cm^2 (Milkiewicz et al., 2001). A mini-osmotic pump (Alzet, Cupertino, CA) was inserted surgically into the animal which dispensed p38 inhibitor SB203580 (Tocris, 1 μg/μl in saline, 0.5 μl/h) to the right EDL and extensor hallucis proprius (EHP) for the duration of the experiment. SB202474 (Calbiochem, 1 μg/μl in saline, 0.5 μl/h), an inactive analog of SB203580 was used as a control. The EDL and EHP were extracted on the seventh day of treatment. Surgical procedures were carried out under anesthesia (intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg)), in accordance with Animal Care Procedures at York University and in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Immunostaining

Extracted EDL muscles were snap frozen in cold isopentane and then placed in liquid nitrogen. Ten micrometer frozen cross-sections of EDL muscle were mounted on glass slides and fixed in cold acetone for 10 sec and then blocked in 5% goat serum and 0.05% Triton X-100 in PBS for 1 h. Sections were incubated with phospho-p38 antibody (1:250, Cell Signaling, Beverly, MA) followed by goat-anti-rabbit Alexa 568 (1:400, Molecular Probes, Eugene, OR) and fluorescein griffonia simplicifolia lectin I (1:400, Vector Laboratories, Burlingame, CA). Slides were mounted with glass cover slips using Aquaperm mounting medium (Thermo Scientific, Ottawa, ON). Muscle sections were examined using confocal microscopy (Olympus Fluoview 300, Argon laser, 488 nm, HeNe laser, 543 nm, pinhole aperture 2). Olympus Fluoview software was used to capture a z-series (0.08 μm steps). Images are presented as a stack of 10 images.

Electron Microscopy

The EHP, a thin muscle appropriate for electron microscopy (Zhou et al., 1998) which also received drug delivery, was fixed in situ, and then removed and prepared by standard transmission electron microscopy protocol. Sections were viewed using a Philips EM201 electron microscope.

Western Blot

Protein from cell culture and muscle homogenate was extracted by lysis with RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF supplemented with 10% protease inhibitor cocktail, 1 mM sodium orthovanadate, and 1 mM sodium fluoride. Protein from media used to shear the cells was concentrated with Millipore (Etobicoke, ON) Amicon Ultra 10K concentrators as directed by the manufacturer. Quantification of protein was determined by micro bicinchonic acid assay (Thermo Scientific) as directed by the manufacturer. Thirty micrograms of protein was prepared in a reducing sample buffer, denatured, and run through a 7% or 12% SDS–polyacrylamide separating gel. Followed by semi-dry transfer or wet transfer onto a PVDF membrane (Immobilon-P, Millipore). The membrane was then blocked with 5% milk in TBS supplemented with 0.1% Tween-20 (TTBS) and incubated overnight with anti-VEGFR2 (phospho-Y1214; 1:250, Abcam, Cambridge, MA), anti-phospho-ERK1/2 antibody (1:1,000, Cell Signaling), anti-phospho-p38 antibody (1:1,000 dilution, Cell Signaling), anti-phospho-Akt (1:1,000, Cell Signaling), or anti-VEGF (1:200, SC-152, Santa Cruz Biotechnology, Santa Cruz, CA) in a 5% BSA diluted in TTBS. A rabbit IgG HRP-linked secondary antibody incubation (1:10,000, GE Biosciences, Uppsala, SE) was performed, followed by a chemiluminescent reaction (chemiluminescent HRP substrate, Millipore) and exposed to film. Bands were quantified by densitometry (FluorChem, Alpha Innotech Corporation, San Leandro, CA). For loading normalization, the membrane was then stripped at 50°C for 15 min in Western stripping buffer, and was followed by washes in TTBS. The membrane was then re-probed

with anti-FLK1 (1:500, Santa Cruz Biotechnology), p38, Akt, β-actin, or tubulin antibody (1:1,000, Cell Signaling).

Muscle Capillarity Determination

Muscle capillarity was determined by the capillary to muscle fiber ratio. Ten micrometer cross-sections of the EDL were stained to identify capillaries using BCIP/NBT (Sigma) for visualization of alkaline phosphatase and sections were stained with Ponceau to enhance muscle fiber contrast. Cross-sections were viewed with an Olympus microscope (20× objective). In each field of view, capillaries and muscle fibers were counted and expressed as a ratio of capillaries/muscle fibers (C/F). Average C/F was calculated from five independent fields of view per rat. An increased ratio is indicative of enhanced angiogenesis.

Statistical Analysis

The graphs are presented as amount of protein relative to control ± SE. Single comparisons were assessed with paired *t*-tests. Multiple comparisons with one variable were made with the one-way ANOVA followed by Tukey or Fisher's protected least significant difference (PLSD) post hoc tests. Two-way ANOVA was used for multiple comparisons with two variables. Significance was set at $\alpha = 0.05$. GraphPad Prism 4.0 software package was used to perform statistical analyses.

Results

The VEGFR2 Regulates Shear Stress-Induced p38 Phosphorylation

We assessed VEGFR2 phosphorylation on Y1214 following shear stress exposure. Y1214 phosphorylation was elevated by 30 min and returned to basal levels at 2 h (Fig. 1A). Our group previously demonstrated that p38 phosphorylation is increased in cells exposed to 2 h of shear stress (Milkiewicz et al., 2006). To determine if VEGFR2 contributes to this activation, cells were exposed to shear stress for 2 or 6 h in the presence or absence of VEGFR2i. p38 phosphorylation was increased in response to shear stress, the shear stress-induced increase was abolished with VEGFR2i treatment at both 2 and 6 h (Fig. 1B,C). Efficacy of the VEGFR2i in this experiment was confirmed through analysis of VEGFR2 phosphorylation on Y1214 in cells treated with 10 μM VEGFR2i and stimulated with VEGF. VEGFR2 phosphorylation was reduced by the inhibitor (Fig. 1D). Stimulation of cells with VEGF also significantly increased VEGFR2 Y1214 phosphorylation (Fig. 2A) as well as p38 phosphorylation (Fig. 2B).

The Effect of Nitric Oxide Synthase Inhibition on p38 Phosphorylation in Shear Stress

Following 6 h shear stress exposure p38 phosphorylation was elevated, but NOS inhibition had no effect on the increase in p38 phosphorylation (Fig. 3A), which is similar to our observation for 2 h shear stress exposure in the presence of NOS inhibitor (Milkiewicz et al., 2006). p38 phosphorylation remained elevated after 24 h shear stress versus static control (Fig. 3B).

The Role of p38 in Shear Stress-Mediated VEGF Production

Previous studies reported that elevated shear stress causes increased VEGF production in microvascular endothelial cells (Milkiewicz et al., 2001; Rivilis et al., 2002). We investigated shear stress-induced VEGF secretion by Western blot analysis of conditioned media from static or shear-stimulated cells. After 6 h of shear stress exposure, VEGF secretion increased over threefold compared to the static control (Fig. 4A). We also assessed the role of p38 in VEGF protein production in cell lysates in response to shear stress. VEGF protein increased

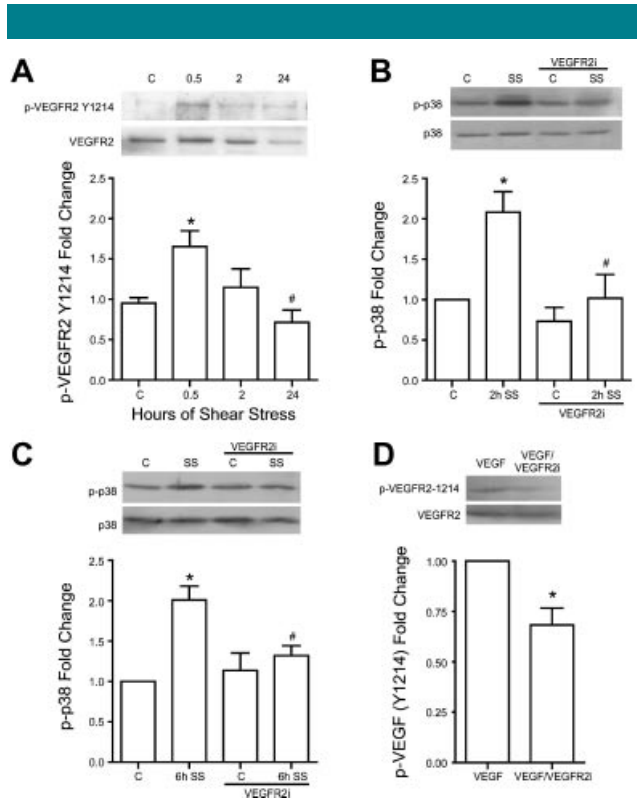

Fig. 1. Involvement of shear stress (SS)-induced vascular endothelial growth factor receptor 2 (VEGFR2) activation in p38 phosphorylation. Cells were sheared at 12 dynes/cm^2 in the presence or absence of 10 μM VEGF receptor 2 kinase inhibitor I (VEGFR2i). Western blotting of cell extracts was performed for (A) VEGFR2-Y1214 phosphorylation (fold change normalized to total VEGFR2 levels) ($^*P < 0.05$ vs. control (C), $^\#P < 0.05$ vs. 0.5 h SS; n = 4); (B) phosphorylated p38 (fold change normalized to total p38 levels) following 2 h of shear stress ($^*P < 0.05$ vs. C, $^\#P < 0.05$ vs. SS; n = 5); and (C) and 6 h of shear stress ($^*P < 0.05$ vs. C, $^\#P < 0.05$ vs. SS; n = 3). D: VEGFR2i efficacy was assessed by VEGFR2 (Y1214) phosphorylation in response to VEGF stimulation and VEGFR2i treatment ($^*P < 0.05$ vs. VEGF, n = 3). Values are means ± SE.

following 6 h of elevated shear stress and this response was abolished by administration of 10 μM SB203580 (Fig. 4B). Akt phosphorylation was unaffected by the use of 10 μM SB203580 (Supplemental Fig. 1).

Effects of VEGFR2 and NOS Inhibition on Shear Stress-Induced ERK1/2 Phosphorylation

ERK1/2 phosphorylation also increases in cells exposed to 2 h of shear stress (Milkiewicz et al., 2006). However, this increase in ERK1/2 phosphorylation was unaffected by VEGFR2i treatment (Fig. 5A). In contrast, VEGF stimulation of static endothelial cells resulted in ERK1/2 phosphorylation (Fig. 5B). ERK1/2 phosphorylation was no longer elevated at 6 h of shear stress (Fig. 5C) and remained at basal levels following 24 h of shear stress (Fig. 5D).

Enhanced Shear Stress Induces p38-Mediated Luminal Splitting In Vivo

Because of the dominant and sustained activation of p38, we focused on examining the involvement of p38 activity during shear stress stimulation in vivo. Chronically elevated shear stress was induced in vivo through administration of the vasodilator prazosin, as previously described by Ziada et al.

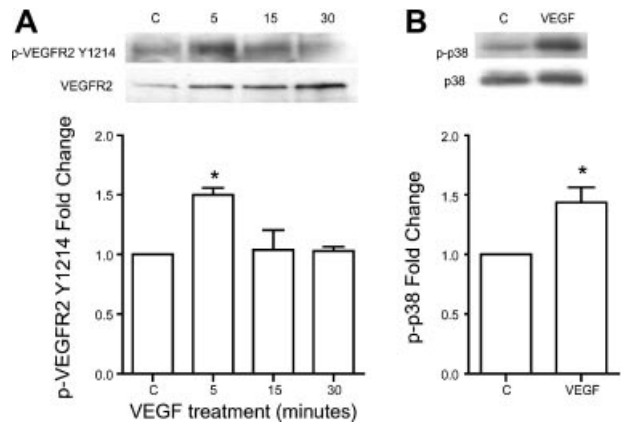

Fig. 2. Vascular endothelial growth factor receptor 2 (VEGFR2)-Y1214 and p38 phosphorylation in response to vascular endothelial growth factor (VEGF) stimulation. Cells were treated with recombinant VEGF (20 ng/ml) for 5, 15, or 30 min prior to protein extraction. Western blotting was performed for (A) VEGFR2-Y1214 phosphorylation (fold change normalized to total VEGFR2 levels) (Fisher's PLSD, $^{*}P < 0.05$ vs. control (C), n = 4) and (B) phosphorylated p38 following 30 min VEGF stimulation (fold change normalized to total p38) ($^{*}P < 0.05$ vs. C; n = 4). Values are means ± SE.

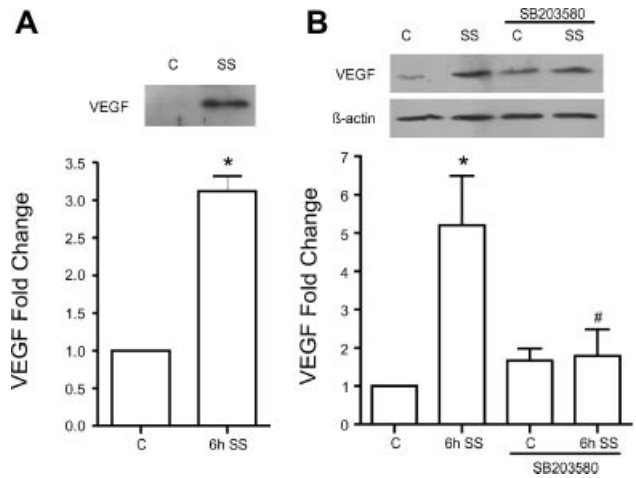

Fig. 4. VEGF production in response to shear stress (SS) and SB203580. Cells were sheared for 6 h in the presence or absence of p38 inhibitor (10 μM SB203580). A: Media used to shear the cells ($^{*}P < 0.05$ vs. C; n = 3) and (B) cell lysates were analyzed for VEGF-A protein by Western blotting (fold change normalized to β-actin) ($^{*}P < 0.05$ vs. C, $^{\#}P < 0.05$ vs. SS; n = 3). Values are means ± SE.

(1989) and Milkiewicz et al. (2001). p38 phosphorylation was measured in EDL muscle after 1, 2, 4, or 7 days of treatment. p38 phosphorylation was elevated significantly by prazosin treatment at 2 days of treatment (Fig. 6A) and returned to basal levels by 7 days (Fig. 6B). Immunoreactivity for phosphorylated p38 was strongest in the capillaries from EDL of 2-day prazosin-treated rats as compared to that seen in non-treated and 1-, 4-, and 7-day prazosin-treated rats (Fig. 7).

The role of p38 MAPK in prazosin-induced angiogenesis was assessed following administration of the p38 inhibitor SB203580, or its inactive analog SB202474, in combination with 7-day prazosin treatment. We assessed levels of p38α phosphorylation to verify drug efficacy, as it was reported that p38α phosphorylation is sensitive to SB203580 inhibition of p38 activity (Ge et al., 2002). p38α phosphorylation was significantly lower in muscle extracts from the SB203580-treated animals compared to those from rats treated with the inactive analog SB202474 (Supplemental Fig. 2). Cross-sections from the EHP were examined by electron microscopy. Both the control and SB203580-treated capillaries exhibited a quiescent phenotype without pronounced luminal projections or vesicles (Fig. 8A,C). Capillaries from 7-day prazosin treatment showed formation of numerous projections into the lumen, and enhanced vesicle production throughout the endothelium, as previously reported by Zhou et al. (1998) (Fig. 8B). In contrast, p38 inhibition abolished shear stress-induced luminal projection and vesicle formation in prazosin-treated rats (Fig. 8D).

Consistent with the findings by Zhou et al., prazosin induced an increase in the capillary to muscle fiber ratio, which indicates formation of new capillaries (Fig. 8E) (Zhou et al., 1998). SB203580 treatment itself did not affect the capillary to muscle fiber ratio, however, the prazosin-induced increase in the capillary to muscle fiber ratio was abolished in SB203580-treated animals.

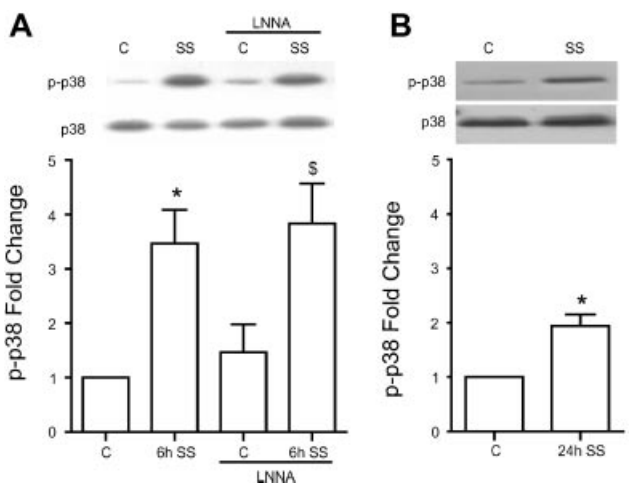

Fig. 3. Effect of long duration shear stress (SS) and nitric oxide synthase (NOS) inhibition on p38 phosphorylation. Cells were sheared for 6 or 24 h in the presence or absence of the NOS inhibitor, 30 μM omega *N*-nitro-L-arginine (LNNA). Western blotting for (A) phosphorylated p38 after 6 h (fold change normalized to total p38 levels) ($^{*}P < 0.05$ vs. control (C), $^{\$}P < 0.05$ vs. C+LNNA; n = 4) and (B) phosphorylated p38 after 24 h (fold change normalized to total p38) ($^{*}P < 0.05$ vs. C; n = 3). Values are means ± SE.

Discussion

The present study demonstrated that shear stress-mediated angiogenesis requires p38 activity. Shear stress induced a substantial and sustained elevation in p38 phosphorylation. SB203580 treatment abolished prazosin-induced luminal projections and reduced vacuole formation. We also report that VEGFR2 activation is necessary for p38 phosphorylation in response to shear stress in vitro.

We found that the VEGFR2 kinase I inhibitor significantly reduced p38 phosphorylation in response to shear stress. Supporting the role of VEGFR2 in p38 phosphorylation, Shay-Salit et al. (2002) found that shear stress caused a rapid (1–2 min) interaction between VEGFR2 and VE-cadherin that was needed for p38 phosphorylation. Other researchers have

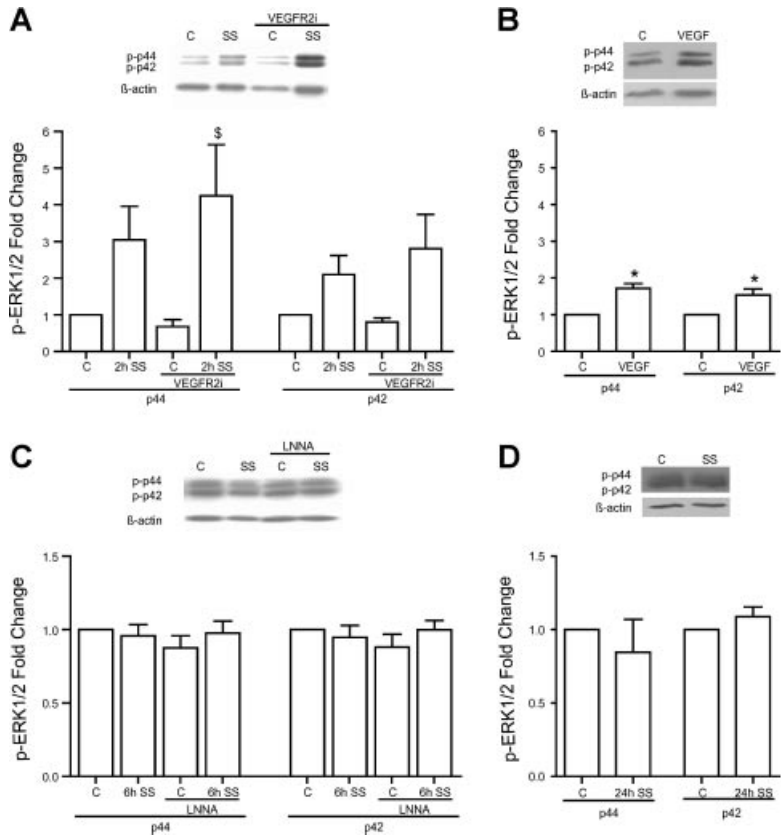

Fig. 5. The effects of VEGFR2 activation and NOS inhibition on ERK1/2 phosphorylation in response to shear stress (SS). Western blotting of cell extracts was performed for ERK1/2 phosphorylation (fold change normalized to β-actin) on cells (A) sheared in the presence or absence of 10 μM VEGFR2i for 2 h ($^{\$}P < 0.05$ vs. C + VEGFR2i; n = 5), (B) plated in OptiMem (low serum media) and treated with recombinant VEGF (20 ng/ml) for 30 min ($^{*}P < 0.05$ vs. C; n = 3) and (C) sheared in the presence or absence of 30 μM LNNA for 6 h (NS; n = 3) and (D) following 24 h of shear stress (NS; n = 3). Values are means ± SE.

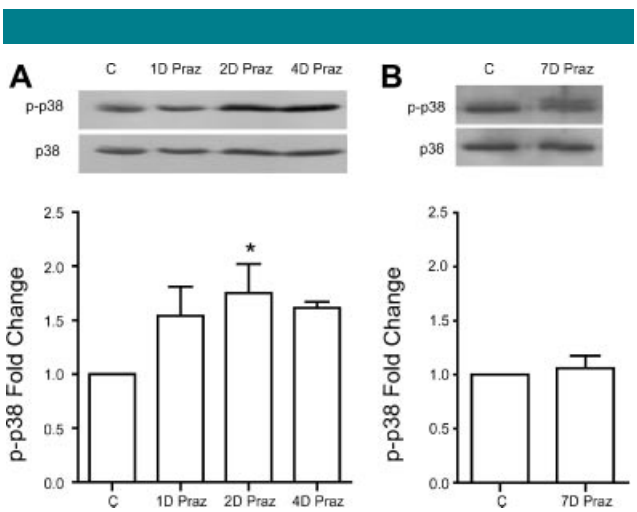

Fig. 6. p38 phosphorylation in vivo in response to prazosin treatment. Prazosin (Praz), an alpha1-adrenergic receptor inhibitor, was administered to male Sprague–Dawley rats for up to 7 days (7D). Protein extracts from EDL muscles were analyzed for phosphorylated p38 by Western blotting. A: phosphorylated p38 after 1, 2, or 4 days (4D) Praz (fold change normalized to total p38) ($^{*}P < 0.05$ vs. C, n = 4). B: Phosphorylated p38 after 7 days (7D) Praz (fold change normalized to total p38) (NS, n = 4). Values are means ± SE.

shown that Y1214 on VEGFR2 regulates the phosphorylation of p38 (Lamalice et al., 2004). Consistent with the findings, we observed phosphorylation of Y1214 in response to shear stress.

This study revealed that p38 phosphorylation is prolonged in response to shear stress exposure, remaining significantly elevated at 2, 6, and 24 h. Although NOS inhibition has been reported to abolish shear stress-mediated angiogenesis (Baum et al., 2004), inhibition of NOS activity did not affect p38 phosphorylation in response to shear stress. NO signaling may lie downstream of p38 (Anter et al., 2005) or be part of a distinct signaling pathway. In contrast to p38, increased ERK1/2 phosphorylation was constrained temporally, as it was elevated at 2 h but returned to basal levels at 6 or 24 h, suggesting that p38 signaling predominates over ERK1/2 during sustained elevated shear stress. Interestingly, we found that VEGFR2 inhibition did not attenuate the activation of ERK1/2 in response to 2 h of shear stress. This suggests that VEGFR2 activation by shear stress differs from VEGF-A stimulation of the receptor, which did result in ERK1/2 phosphorylation.

The sustained involvement of p38, rather than ERK1/2, signaling in response to elevated shear stress is consistent with the cellular and morphological events known to occur with chronic shear stress-mediated angiogenesis. Shear stress-induced angiogenesis does not involve basement membrane degradation (Rivilis et al., 2002). We reported previously that basement membrane degrading enzyme, matrix metalloproteinase-2 (MMP-2), is down-regulated by shear stress in a p38-dependent manner (Milkiewicz et al., 2006).

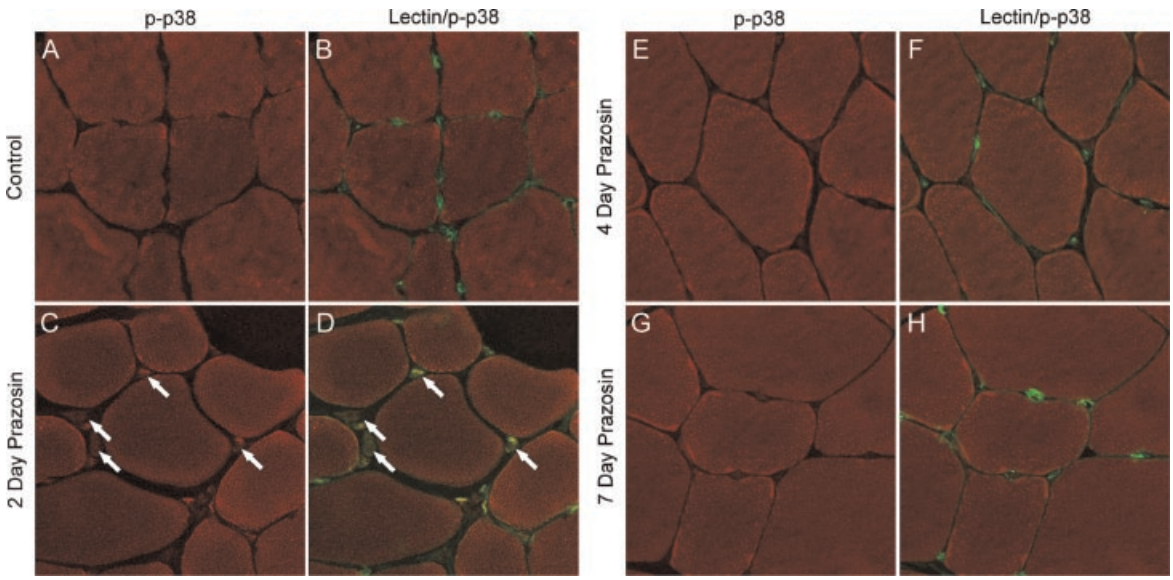

Fig. 7. Effect of prazosin treatment on p38 phosphorylation in the capillaries of the EDL. Prazosin was administered to male Sprague–Dawley rats for up to 7 days. EDL muscle cross-sections were stained with anti-phospho-p38 (red) and counterstained with fluorescein griffonia simplicifolia lectin I (Vector Laboratories) (green) to visualize capillaries. Sections were viewed by confocal microscopy (Olympus Fluoview 300, z-series, 10 slices). A: phospho-p38 and (B) lectin/phospho-p38 overlay of a muscle section of an untreated rat. C: phospho-p38 and (D) lectin/phospho-p38 staining overlay of a muscle section of a 2-day prazosin-treated animal. Arrows indicate sites of p38 phosphorylation co-localized to capillaries. E: phospho-p38 and (F) lectin/phospho-p38 staining overlay of a muscle section of a 4-day prazosin-treated rat. G: phospho-p38 and (H) lectin/phospho-p38 staining overlay of a muscle section of a 7-day prazosin-treated animal.

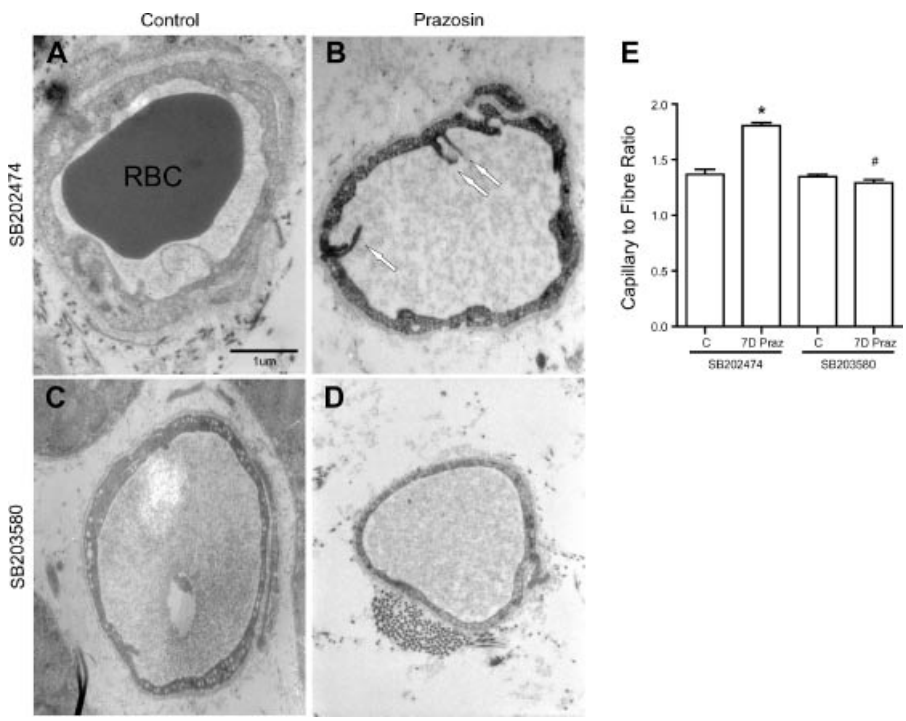

Fig. 8. Capillary morphology and muscle capillarity in response to prazosin and SB203580 treatment. Prazosin (Praz) and the p38 inhibitor SB203580, or its inactive analog SB202474, was administered to male Sprague–Dawley rats for 7 days (D) by mini-osmotic pump (1 μg/μl, 0.5 μl/h). The extensor hallucis proprius (EHP) and EDL from each rat were extracted and prepared for electron microscopy or muscle capillarity analysis respectively. Electron microscopy of the EHP revealed (A) a quiescent state of the capillary in the control animal (RBC, red blood cell). B: Prazosin treatment induces luminal projections (arrows) and pronounced vesicles throughout the endothelium. C: p38 inhibition resembles the control (D) p38 inhibition and prazosin treatment also resemble the control, with the absence of luminal projections. The scale bar represents 1 μm and is equivalent for each panel. E: Capillary to muscle fiber ratio in the EDL ($^{*}P < 0.05$ vs. control (C) + SB202474, $^{\#}P < 0.05$ vs. shear stress (SS) + SB202474; n = 4). Values are means ± SE.

Conversely, ERK1/2 activation increases MMP-2 production (Boyd et al., 2005; Mountain et al., 2007). ERK1/2 also promotes proliferation in endothelial cells (Pages et al., 1993; Pages et al., 2000), and minimal proliferation occurs during shear stress-mediated angiogenesis (Zhou et al., 1998).

We also observed an increase in p38 phosphorylation in capillaries in response to prazosin stimulation. Immunostaining showed intense localization of phosphorylated p38 to the capillaries of 2-day prazosin treated muscles, but minimal signal at later time points. This finding supports a role for p38 activation as an early response to shear stress, occurring prior to the formation of new capillaries.

Our data support the hypothesis that shear stress-induced p38 phosphorylation initiates signals required for shear stress-mediated angiogenesis, as p38 inhibition prevented the typical increase in capillary to muscle fiber ratio observed with prazosin treatment. It is notable that VEGF-A mRNA increases in cultured microvascular endothelial cells exposed to shear stress (Milkiewicz et al., 2007) and that VEGF-A protein levels are elevated in vivo after 2 days of prazosin treatment (Milkiewicz et al., 2001; Rivilis et al., 2002). In agreement, we found that VEGF-A secretion by cultured microvascular endothelial cells increases substantially in response to shear stress exposure. We also observed substantial increases in VEGF-A protein levels in cell lysates in response to shear stress stimulation in vitro, which were abrogated by inhibition of p38 activity. Regulation of VEGF production by p38 was also reported to occur in mouse podocytes (Kang et al., 2006) and in glioma cells (Yoshino et al., 2006). Thus, p38-dependent production of VEGF-A may be a critical step in the process of shear stress-induced angiogenesis. Consistent with this possibility, administration of VEGF trap prevented the shear stress-mediated morphological changes of the formation of vacuoles, filopodia (luminal projections) and the increase in capillary to muscle fiber ratio in response to prazosin (Williams et al., 2006), similar to the effects we observed following p38 inhibition.

In conclusion, our data support a positive role for p38 activation in shear stress-mediated angiogenesis. A similar role for p38 was reported by Rocic et al. (2007), as they found that p38 inhibition prevented both VEGF induced tube formation in cell culture and significantly reduced ischemia/reperfusion induced coronary collateral formation in vivo. Here we show that shear stress induces VEGFR2 phosphorylation on Y1214 and that VEGFR2 activation is essential for elevated p38 phosphorylation. Our study has identified a novel role for p38 in mediating signals required for shear stress-induced angiogenesis, including production of VEGF-A. This study is an important step in identifying signaling events that contribute to shear stress-mediated capillary remodeling.

Acknowledgments

The authors thank Karen Rethoret for TEM specimen processing and assistance with micrograph development and Damiano Serio for assistance with ERK1/2 Western blots. Funding for this research project was provided by CIHR and HSF Canada (T.L.H.). Support was also provided to E.G. from NSERC PGS D scholarship.

Literature Cited

Anter E, Chen K, Shapira OM, Karas RH, Keaney JF, Jr. 2005. p38 mitogen-activated protein kinase activates eNOS in endothelial cells by an estrogen receptor alpha-dependent pathway in response to black tea polyphenols. Circ Res 96:1072–1078.

Baum O, Da Silva-Azevedo L, Willerding G, Wockel A, Planitzer G, Gossrau R, Pries AR, Zakrzewicz A. 2004. Endothelial NOS is main mediator for shear stress-dependent angiogenesis in skeletal muscle after prazosin administration. Am J Physiol Heart Circ Physiol 287:H2300–H2308.

Boyd PJ, Doyle J, Gee E, Pallan S, Haas TL. 2005. MAPK signaling regulates endothelial cell assembly into networks and expression of MT1-MMP and MMP-2. Am J Physiol Cell Physiol 288:C659–C668.

Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, Shyy JY. 1999. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. J Biol Chem 274:18393–18400.

Chien S. 2007. Mechanotransduction and endothelial cell homeostasis: The wisdom of the cell. Am J Physiol Heart Circ Physiol 292:H1209–H1224.

Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J. 2002. MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295:1291–1294.

Gomes E, Rockwell P. 2008. p38 MAPK as a negative regulator of VEGF/VEGFR2 signaling pathway in serum deprived human SK-N-SH neuroblastoma cells. Neurosci Lett 431:95–100.

Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa WC. 2001. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem 276:30359–30365.

Han X, Boyd PJ, Colgan S, Madri JA, Haas TL. 2003. Transcriptional up-regulation of endothelial cell matrix metalloproteinase-2 in response to extracellular cues involves GATA-2. J Biol Chem 278:47785–47791.

Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. 2003. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res 93:354–363.

Jin ZG, Wong C, Wu J, Berk BC. 2005. Flow shear stress stimulates Gab1 tyrosine phosphorylation to mediate protein kinase B and endothelial nitric-oxide synthase activation in endothelial cells. J Biol Chem 280:12305–12309.

Kang YS, Park YG, Kim BK, Han SY, Jee YH, Han KH, Lee MH, Song HK, Cha DR, Kang SW, Han DS. 2006. Angiotensin II stimulates the synthesis of vascular endothelial growth factor through the p38 mitogen activated protein kinase pathway in cultured mouse podocytes. J Mol Endocrinol 36:377–388.

Kanno S, Oda N, Abe M, Terai Y, Ito M, Shitara K, Tabayashi K, Shibuya M, Sato Y. 2000. Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. Oncogene 19:2138–2146.

Lamalice L, Houle F, Jourdan G, Huot J. 2004. Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. Oncogene 23:434–445.

Le Boeuf F, Houle F, Huot J. 2004. Regulation of vascular endothelial growth factor receptor 2-mediated phosphorylation of focal adhesion kinase by heat shock protein 90 and Src kinase activities. J Biol Chem 279:39175–39185.

Li YS, Haga JH, Chien S. 2005. Molecular basis of the effects of shear stress on vascular endothelial cells. J Biomech 38:1949–1971.

Milkiewicz M, Brown MD, Egginton S, Hudlicka O. 2001. Association between shear stress, angiogenesis, and VEGF in skeletal muscles in vivo. Microcirculation 8:229–241.

Milkiewicz M, Kelland C, Colgan S, Haas TL. 2006. Nitric oxide and p38 MAP kinase mediate shear stress-dependent inhibition of MMP-2 production in microvascular endothelial cells. J Cell Physiol 208:229–237.

Milkiewicz M, Doyle JL, Fudalewski T, Ispanovic E, Aghasi M, Haas TL. 2007. HIF-1alpha and HIF-2alpha play a central role in stretch-induced but not shear-stress-induced angiogenesis in rat skeletal muscle. J Physiol 583:753–766.

Milkiewicz M, Uchida C, Gee E, Fudalewski T, Haas TL. 2008. Shear stress-induced Ets-1 modulates protease inhibitor expression in microvascular endothelial cells. J Cell Physiol 217:502–510.

Mountain DJ, Singh M, Menon B, Singh K. 2007. Interleukin-1beta increases expression and activity of matrix metalloproteinase-2 in cardiac microvascular endothelial cells: Role of PKCalpha/beta1 and MAPKs. Am J Physiol Cell Physiol 292:C867–C875.

Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM. 2000. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci USA 97:10454–10459.

Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouyssegur J. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci USA 90:8319–8323.

Pages G, Milanini J, Richard DE, Berra E, Gothie E, Vinals F, Pouyssegur J. 2000. Signaling angiogenesis via p42/p44 MAP kinase cascade. Ann N Y Acad Sci 902:187–200.

Resnick N, Yahav H, Shay-Salit A, Shushy M, Schubert S, Zilberman LC, Wofovitz E. 2003. Fluid shear stress and the vascular endothelium: For better and for worse. Prog Biophys Mol Biol 81:177–199.

Rivilis I, Milkiewicz M, Boyd P, Goldstein J, Brown MD, Egginton S, Hansen FM, Hudlicka O, Haas TL. 2002. Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis. Am J Physiol Heart Circ Physiol 283:H1430–H1438.

Rocic P, Kolz C, Reed R, Potter B, Chilian WM. 2007. Optimal reactive oxygen species concentration and p38 MAP kinase are required for coronary collateral growth. Am J Physiol Heart Circ Physiol 292:H2729–H2736.

Shay-Salit A, Shushy M, Wolfovitz E, Yahav H, Breviario F, Dejana E, Resnick N. 2002. VEGF receptor 2 and the adherens junction as a mechanical transducer in vascular endothelial cells. Proc Natl Acad Sci USA 99:9462–9467.

Traub O, Berk BC. 1998. Laminar shear stress: Mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol 18:677–685.

Williams JL, Cartland D, Rudge JS, Egginton S. 2006. VEGF trap abolishes shear stress- and overload-dependent angiogenesis in skeletal muscle. Microcirculation 13:499–509.

Yang S, Xin X, Zlot C, Ingle G, Fuh G, Li B, Moffat B, de Vos AM, Gerritsen ME. 2001. Vascular endothelial cell growth factor-driven endothelial tube formation is mediated by vascular endothelial cell growth factor receptor-2, a kinase insert domain-containing receptor. Arterioscler Thromb Vasc Biol 21:1934–1940.

Yoshino Y, Aoyagi M, Tamaki M, Duan L, Morimoto T, Ohno K. 2006. Activation of p38 MAPK and/or JNK contributes to increased levels of VEGF secretion in human malignant glioma cells. Int J Oncol 29:981–987.

Zhou A, Egginton S, Hudlicka O, Brown MD. 1998. Internal division of capillaries in rat skeletal muscle in response to chronic vasodilator treatment with alpha1-antagonist prazosin. Cell Tissue Res 293:293–303.

Ziada A, Hudlicka O, Tyler KR. 1989. The effect of long-term administration of alpha 1-blocker prazosin on capillary density in cardiac and skeletal muscle. Pflugers Arch 415:355–360.