p38 MAPK is Activated by Vascular endothelial growth factor receptor 2 and is essential for shear stress-induced angiogenesis

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Abstract

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/cm², 0.5–24 hours). VEGFR2-Y1214 phosphorylation increased in response to elevated shear stress and VEGF stimulation. p38 and ERK1/2 phosphorylation increased at 2 hours of shear stress, but only p38 remained phosphorylated at 6 and 24 hours of shear stress. VEGFR2 inhibition abrogated p38, but not ERK1/2 phosphorylation. VEGF production was increased in response to shear stress at 6 hours, 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 fibre ratio seen after 7 days of prazosin treatment. Our data suggest that p38 activation is necessary for shear stress-dependent angiogenesis.

Keywords

mitogen activated protein kinase; luminal splitting; mechanotransduction; VEGF

Introduction

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- κ B, 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 (Lamallice 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 (Chien, 2007; Milkiewicz et al., 2006). Sequestration of VEGF or inhibition of nitric oxide synthase (NOS) activity abolishes shear stress-mediated luminal projection formation and angiogenesis (Williams et al., 2006; Baum et al., 2004).

Shear stress-dependent interaction of vascular endothelial (VE)-cadherin with VEGFR2 occurs within 1 to 2 minutes 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 (Rocic et al., 2007; Yang et al., 2001). Gratton et al. reported that p38 depresses Akt levels resulting in apoptotic signaling (Gratton et al., 2001). Similarly, serum starvation induced apoptosis in neuronal cells that was mediated by p38 (Gomes and Rockwell, 2008). However, p38 α deletion results in homozygous embryonic lethality due to reduced 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.

Materials and 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 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% Glutamax (Invitrogen), 0.11 mg/ml sodium pyruvate) at 7% CO₂ 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. (Milkiewicz et al., 2006) for 0.5, 2, 6 or 24 hours. 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) connected to parallel laminar closed flow chambers (FCS2, Bioprocess) was used to pump media generating shear stress of 12 dynes/cm². VEGFR2 was inhibited using 10 µM VEGFR2 kinase inhibitor I (VEGFR2i) (Calbiochem 676480). Efficacy of VEGFR2i inhibition of VEGFR2 (Y1214) phosphorylation was assessed in pre-treated cells followed by 5 minute 20 ng/ml VEGF (Cedarlane Laboratories) stimulation by Western blot. ERK1/2 and p38 phosphorylation was measured after cells were stimulated with 20 ng/ml of VEGF for 30 minutes. To inhibit eNOS, cells were treated with 30 µM Omega N-nitro-L-arginine (LNNA) (Sigma) (Milkiewicz et al., 2006). Cells were pretreated with the inhibitors for 1 hour prior to shear stress exposure.

In Vivo Shear Stress

Male Sprague-Dawley rats (150–200 grams) were administered prazosin (50 mg/L drinking water, ad libitum), an alpha₁-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/cm² (Milkiewicz et al., 2001). A mini-osmotic pump (Alzet) was inserted surgically into the animal which dispensed p38 inhibitor SB203580 (Tocris, 1 µg/µl in saline, 0.5 µl/hr) 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/hr), an inactive analog of SB203580 was used as a control. The EDL and EHP were extracted on the 7th day of treatment. Surgical procedures were carried out under anesthesia (intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10mg/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 then placed in liquid nitrogen. 10 μ m frozen cross-sections of EDL muscle were mounted on glass slides and fixed in cold acetone for 10 seconds then blocked in 5% goat serum and 0.05% Triton X-100 in PBS for one hour. Sections were incubated with phospho-p38 antibody (1:250, Cell Signaling) followed by goat-anti-rabbit Alexa 568 (1:400, Molecular Probes) and fluorescein griffonia simplicifolia lectin I (1:400, Vector Laboratories). Slides were mounted with glass cover slips using Aquaperm mounting medium (Thermo Scientific). 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 μ m 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*, 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. Quantification of protein was determined by micro bicinchonic acid assay (Thermo Scientific) as directed by the manufacturer. 30 μ g 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), anti-phospho-ERK1/2 antibody (1:1000, Cell Signaling), anti-phospho-p38 antibody (1:1000 dilution, Cell Signaling), or anti-VEGF (1:200, SC-152, Santa Cruz Biotechnology) in a 5% BSA diluted in TTBS. A rabbit IgG HRP linked secondary antibody incubation (1:10000, GE Biosciences) was performed, followed by a chemiluminescent reaction (Chemiluminescent HRP substrate, Millipore) and exposed to film. Bands were quantified by densitometry (FluorChem, Alpha Innotech Corporation). For loading normalization, the membrane was then stripped at 50 °C for 15 minutes 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, β -actin or tubulin antibody (1:1000, Cell Signaling).

Muscle Capillarity Determination

Muscle capillarity was determined by the capillary to muscle fibre ratio. 10 μ m 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 fibre contrast. Cross sections were viewed with an Olympus microscope (20x objective). In each field of view, capillaries and muscle fibres were counted and expressed as a ratio of capillaries:muscle fibres (C:F). Average C:F were 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 $\pm$ 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 2 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 minutes and returned to basal levels at 2 hours (Figure 1A). Our group previously demonstrated that p38 phosphorylation is increased in cells exposed to 2 hours 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 hours 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 hours (Figure 1B, C). Efficacy of the VEGFR2i in this experiment was confirmed through analysis of VEGFR2 phosphorylation on Y1214 in cells treated with 10 μ M VEGFR2i and stimulated with VEGF. VEGFR2 phosphorylation was reduced by the inhibitor (Figure 1D). Stimulation of cells with VEGF also significantly increased VEGFR2 Y1214 phosphorylation (Figure 2A) as well as p38 phosphorylation (Figure 2B).

The effect of nitric oxide synthase inhibition on p38 phosphorylation in shear stress

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

The role of p38 in shear stress-mediated VEGF production

Previous studies reported that elevated shear stress causes increased VEGF production (Milkiewicz et al., 2001; Rivilis et al., 2002). We assessed the role of p38 in VEGF protein

production in response to shear stress. VEGF protein increased following 6 hours of elevated shear stress and this response was abolished by administration of SB203580 (Figure 4).

Effects of VEGFR2 and NOS inhibition on shear stress-induced ERK1/2 phosphorylation

ERK1/2 phosphorylation also increases in cells exposed to 2 hours of shear stress (Milkiewicz et al., 2006). However, this increase in ERK1/2 phosphorylation was unaffected by VEGFR2i treatment (Figure 5A). In contrast, VEGF stimulation of static endothelial cells resulted in ERK1/2 phosphorylation (Figure 5B). ERK1/2 phosphorylation was no longer elevated at 6 hours of shear stress (Figure 5C) and remained at basal levels following 24 hours of shear stress (Figure 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 (Ziada et al., 1989) and Milkiewicz (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 (Figure 6A) and returned to basal levels by 7 days (Figure 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 (Figure 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 p38 α phosphorylation to verify drug efficacy, as it was reported that p38 α phosphorylation is sensitive to SB203580 inhibition of p38 activity (Ge et al., 2002). p38 α phosphorylation was significantly lower in muscle extracts from the SB203580 treated animals compared to those from rats treated with the inactive analog SB202474 (Supplemental figure 1). 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 (Figure 8 A, 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. (Zhou et al., 1998) (Figure 8B). In contrast, p38 inhibition abolished shear stress-induced luminal projection and vesicle formation in prazosin treated rats (Figure 8D).

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

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 minute) interaction between VEGFR2 and VE-cadherin that was needed for p38 phosphorylation. Other researchers have shown that Y1214 on VEGFR2 regulates the phosphorylation of p38 (Lamallice 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 hours. 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 hours but returned to basal levels at 6 or 24 hours, 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 hours 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). 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., 2000; Pages et al., 1993), 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 fibre ratio observed with prazosin treatment. It is notable that VEGF-A protein levels are elevated after 2 days of prazosin treatment (Milkiewicz et al., 2001; Rivilis et al., 2002). We observed substantial increases in VEGF-A protein levels in response to shear stress stimulation *in vitro*, which were abrogated by inhibition of p38 activity. Regulation of VEGF production by p38 also was 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 increased capillary to muscle fibre 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 and colleagues, 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* (Rocic et al., 2007). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Involvement of shear stress (SS)-induced vascular endothelial growth factor receptor 2 (VEGFR2) activation in p38 phosphorylation. Cells were sheared at 12 dynes/cm² in the presence or absence of 10 μ M 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 hour SS; n=4); (B) phosphorylated p38 (fold change normalized to total p38 levels) following 2 hours (2h) of shear stress (*P<0.05 vs. C, #P<0.05 vs. SS; n=5); and, (C) and 6 hours (6h) 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 $\pm$ SE.

Figure 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 minutes 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 $\pm$ SE.

Figure 3.

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

Figure 4.

VEGF production in response to shear stress (SS) and SB203580. Cells were sheared for 6 hours (6h) in the presence or absence of p38 inhibitor SB203580. Western blotting for VEGF-A (fold change normalized to β -actin) (* $P < 0.05$ vs. C, # $P < 0.05$ vs. SS; $n = 3$). Values are means $\pm$ SE.

Figure 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 μ M VEGFR2i for 2 hours (2h) ($P < 0.05$ vs. C+VEGFR2i; $n=5$) (B) plated in OptiMem (low serum media) and treated with recombinant VEGF (20 ng/ml) for 30 minutes ($*P < 0.05$ vs. C; $n=3$) (C) sheared in the presence or absence of 30 μ M LNNA for 6 hours (6h) (NS; $n=3$) and (D) following 24 hours (24h) of shear stress (NS; $n=3$). Values are means $\pm$ SE.

Figure 6.

p38 phosphorylation in vivo in response to prazosin treatment. Prazosin (Praz), an α_1 -adrenergic receptor inhibitor, was administered to male Sprague-Dawley rats for up to 7 days (7D). Protein extracts from extensor digitorum longus 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 $\pm$ SE.

Figure 7.

Effect of prazosin treatment on p38 phosphorylation in the capillaries of the extensor digitorum longus (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) p-p38 and (B) lectin/phospho-p38 overlay of a muscle section of an untreated rat. (C) p-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) p-p38 and (F) lectin/phospho-p38 staining overlay of a muscle section of a 4 day prazosin treated rat. (G) p-p38 and (H) lectin/phospho-p38 staining overlay of a muscle section of a 7 day prazosin treated animal.

Figure 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 μ g/ μ l, 0.5 μ l/hour). The extensor hallucis proprius (EHP) and extensor digitorum longus (EDL) from each rat was 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 absence of luminal projections. The scale bar represents 1 μ m, and is equivalent for each panel. (E) Capillary to muscle fibre ratio in the EDL (* P <0.05 vs. control (C)+SB202474, # P <0.05 vs. shear stress (SS)+202474; n =4). Values are means $\pm$ SE.