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Muscle-derived vascular endothelial growth factor regulates microvascular remodelling in response to increased shear stress in mice

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Abstract

Aim: The source of vascular endothelial growth factor-A (VEGF-A) may influence vascular function. Exercise-induced vascular growth has been attributed to elevated metabolic demand and to increased blood flow, involving the production of VEGF-A by skeletal muscle and by endothelial cells respectively. We hypothesized that muscle-derived VEGF-A is not required for vascular adaptations to blood flow in skeletal muscle, as this remodelling stimulus originates within the capillary.

Methods: Myocyte-specific VEGF-A ($mVEGF^{-/-}$) deleted mice were treated for 7–21 days with the vasodilator prazosin to produce a sustained increase in skeletal muscle blood flow.

Results: Capillary number increased in the extensor digitorum longus (EDL) muscle in response to prazosin in wild type but not $mVEGF^{-/-}$mice. Prazosin increased the number of smooth muscle actin-positive blood vessels in the EDL of wild-type but not $mVEGF^{-/-}$ mice. The average size of smooth muscle actin-positive blood vessels also was smaller in knockout mice after prazosin treatment. In response to prazosin treatment, VEGF-A mRNA was elevated within the EDL of wild-type but not $mVEGF^{-/-}$ mice. *Ex vivo* incubation of wild-type EDL with a nitric oxide donor increased VEGF-A mRNA. Likewise, we demonstrated that nitric oxide donor treatment of cultured myoblasts stimulated an increase in VEGF-A mRNA and protein.

Conclusion: These results suggest a link through which flow-mediated endothelial-derived signals may promote myocyte production of VEGF-A. In turn, myocyte-derived VEGF-A is required for appropriate flow-mediated microvascular remodelling. This highlights the importance of the local environment and paracrine interactions in the regulation of tissue perfusion.

Keywords angiogenesis, nitric oxide, skeletal muscle.

Vascular endothelial growth factor-A, or VEGF-A, is a critical growth factor in the initiation and progression of angiogenesis. Initially identified as a protein found in tumour cells that could induce vascular permeability (Senger *et al.* 1983), it was later discovered to be a potent endothelial cell mitogen (Leung *et al.* 1989). In addition to its proliferative and permeability effects, VEGF-A enhances endothelial cell production of proteases, endothelial and smooth muscle cell migration, promotes endothelial cell survival and differentiation and causes arteriolar vasodilation (Pepper *et al.* 1992, Ku *et al.* 1993, Carmeliet *et al.* 1996,

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Grosskreutz *et al.* 1999, Ispanovic *et al.* 2008, Doyle & Haas 2009).

Within skeletal muscle, VEGF-A mRNA increases transiently after a single bout of exercise, returning to baseline levels within a few hours after exercise cessation (Breen *et al.* 1996, Gustafsson *et al.* 1999). Increased levels of soluble VEGF-A protein within the interstitium suggests that VEGF-A secretion from muscle, or release from extracellular matrix, occurs in response to exercise (Hoffner *et al.* 2003). It is thought that skeletal myocytes are the main source of this interstitial VEGF-A (Delavar *et al.* 2014, Hoier & Hellsten 2014).

Skeletal muscle angiogenesis can be stimulated by both metabolic and haemodynamic factors (Egginton *et al.* 2001). However, these stimuli induce capillary growth through different cellular processes. Metabolic stimuli promote the formation of abluminal sprouts that generate lateral branches between adjacent capillaries, while increased shear stress evokes the production of parallel flow pathways through processes of luminal splitting or intussusception, in the absence of abluminal sprouting (Egginton *et al.* 2001, Brown & Hudlická 2003, Djonov *et al.* 2003). VEGF-A has been shown to be critical in regulating angiogenesis regardless of the stimulus (Rivilis *et al.* 2002, Williams *et al.* 2006a). However, it must be recognized that there are multiple cellular sources of VEGF-A within the skeletal muscle (i.e. endothelial cells, myocytes, pericytes) (Leung *et al.* 1989, Darland *et al.* 2003, Bryan *et al.* 2008, Olfert *et al.* 2009). The mechanisms behind exercise-induced vascular growth have been attributed to signals associated with metabolic demand and increased blood flow. The majority of data point to the critical role of myocyte-derived VEGF-A in promoting angiogenesis that occurs in response to elevated metabolic demand. Myocyte-derived VEGF-A is required for the appropriate development of the vasculature in skeletal muscle, as mice with myocyte VEGF-A deleted have approximately 50% reduced capillary to fibre ratio and capillary density compared to wild-type animals (Olfert *et al.* 2009). These mice also fail to undergo skeletal muscle angiogenesis in response to exercise or to muscle overload (Olfert *et al.* 2010, Delavar *et al.* 2014, Gorman *et al.* 2014). Conversely, VEGF-A has been demonstrated to mediate endothelial cell survival through autocrine signalling (Lee *et al.* 2007). Furthermore, endothelial cell VEGF-A production is induced in response to elevated shear stress (Gee *et al.* 2010, dela Paz *et al.* 2012), implicating its involvement in shear stress-mediated vascular remodelling events. Based on these studies, we hypothesized that muscle-derived VEGF-A would not be required for shear stress-stimulated capillary remodelling. We investigated this by analysing the skeletal muscle microvasculature of myocyte-specific VEGF-A deleted mice after sustained exposure to the α-adrenergic receptor antagonist prazosin.

Materials and methods

Chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON) unless otherwise stated.

Ethical approval

Animal studies were approved by the York University Committee on Animal Care and performed in accordance with Animal Care Procedures at York University and the American Physiological Society's Guiding Principles in the Care and Use of Animals.

Animals

Mice harbouring a skeletal myocyte-specific deletion of VEGF-A, in which Cre recombinase is expressed under the control of the muscle creatine kinase promoter, (*skmyCre+:VEGFfl/fl*; herein referred to as mVEGF$^{-/-}$) (Olfert *et al.* 2009) were bred at West Virginia University. Adult mVEGF$^{-/-}$ mice, together with their wild-type littermates (*Cre-:VEGFfl/fl*), were shipped to York University for experiments. mVEGF$^{-/-}$ mice and wild-type (WT) littermates were treated with 50 mg L^{-1} of the α-adrenergic inhibitor prazosin (P7791, Sigma) for 7 or 14 days to increase blood flow to skeletal muscle (Dawson & Hudlická 1989). At the end of each experiment, mice were anesthetized with isoflurane/oxygen (CA2L9108, Baxter Corp., Mississauga, ON, Canada) and the extensor digitorum longus (EDL) muscles were removed, weighed, embedded in OCT (SH75-125D, Fisher Scientific, Fair Lawn, NJ, USA) and frozen in liquid nitrogen and cooled isopentane for histochemistry. Animals were killed by exsanguination.

Muscle and vascular morphometric assessments

To detect capillaries, cryosections of EDL or soleus muscle (12 μm thickness) were fixed in 3.7% formaldehyde and stained with isolectin (fluorescein isothiocyanate–conjugated Griffonia simplicifolia I; Vector Laboratories, Burlington, ON, Canada) diluted in PBS (1 : 100) and Cy-3-conjugated α-smooth muscle actin (C6198, Sigma, 1 : 300) for 60 min. Sections were viewed with a Zeiss Axiovert 200M light microscope (20× objective) equipped with a cooled CCD digital camera (Quantix A01F6020, Photometricx, Tucson, AZ, USA; or, Orca R2 C10600-10B, Hamamatsu, Quorum Technologies Ltd., Guelph, ON, Canada) and images were captured using METAMORPH software version 7.7.8 (Molecular Devices, Sunnyvale, CA, USA). Capillary to muscle fibre ratio (C:F) for each

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mouse was averaged from an unbiased sampling of three or four non-overlapping fields of view (FOV) that each contained ~75–100 muscle fibres. The number of smooth muscle actin-positive (SMA+) vessels was counted within the same FOV used to calculate C:F. SMA+ vessel perimeters were measured using METAMORPH software. In cases where the vessel appeared obliquely or longitudinally oriented, minimal vessel diameter was measured and perimeter was calculated as $P = d\pi$. Myofibre cross-sectional areas were assessed from the same cross sections of EDL and soleus utilized for vascular quantification. Fibre tracings were made using METAMORPH software. A minimum of 100 independent fibres were analysed per mouse.

Electron microscopy

The extensor hallucis proprius (EHP) muscle was utilized for ultrastructural analysis of capillary structure, as this is a fast glycolytic muscle with metabolic and microvascular characteristics similar to the EDL muscle (Myrhage & Hudlická 1976, Hansen-Smith *et al.* 1996), and its thin size is conducive to superfusion-fixation processing for electron microscopy. Mice were anesthetized as described above and the EHP muscle was removed, cut into small sections, and fixed in 3.5% glutaraldehyde for 1 h. Muscle sections were then washed 3 × 15 min. with 0.1 M Na cacodylate and post-fixed in 1% OsO_4 for 1 h. After a serial dehydration in ethanol, sections were incubated in a 50% ethanol, 50% Epon resin mixture overnight. Several muscle fibres were then extracted and embedded in Epon resin for 2 days. The resin block was sectioned and viewed using a Philips EM201 electron microscope at 20 000× magnification. Images were taken using a cooled CCD digital camera (L-3, Scientific Instruments and Applications, Duluth, GA, USA). The following morphological features were quantified within each cross-sectional capillary profile: number of endothelial cells; number of luminal protrusions; number of pericyte associations. In addition, semi-quantitative scoring (−, +, ++) was used to evaluate the presence of cytoplasmic vacuolization and endothelial cell thickening (at non-nuclear regions).

Ex vivo EDL stimulation

EDL muscles from both legs were extracted from four WT mice, and placed in pre-warmed HEPES-saline buffer. The nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) (100 μM; 487910, EMD Biosciences, Darmstadt, Germany) was added to one muscle from each pair, and muscles were incubated at 37 °C. After 2 h, muscles were snap-frozen in liquid nitrogen for subsequent RNA analysis.

Cell culture experiments

C2C12 mouse myoblasts (a gift from Dr. David Hood, York University) were cultured in gelatin-coated flasks with Dulbecco's modified Eagle's medium (DMEM) which was further supplemented with 10% foetal bovine serum (Invitrogen, Burlington, ON, Canada), 50 μg mL^{-1} penicillin/streptomycin, 1% Glutamax (35050-061, Invitrogen) and 1 mM sodium pyruvate (Invitrogen). C2C12 cells were plated to confluence on 35-mm dishes, and then treated overnight with SNAP (10 or 100 μM), or 30 μM of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (LNNA) (N5501, Sigma).

Western blot

C2C12 cells were lysed using Triton lysis buffer (100 mM Tris HCl, 0.1% Triton X-100, 5% glycerol in ddH_2O, pH = 8.7) supplemented with 10% protease inhibitor cocktail and 1.1 μM sodium orthovanadate. Protein extracts of tibialis anterior (TA) muscle were prepared as described previously (Milkiewicz *et al.* 2011). 20 μg of protein per condition, as determined by bicinchonic acid assay (23227, Pierce, Rockford, IL, USA) was prepared in reducing loading buffer, denatured and separated on a SDS-polyacrylamide gel. Protein was transferred onto a PVDF membrane (IPVH00010, Millipore, Billerica, MA, USA) using the wet transfer method (Bio-Rad, Mississauga, ON, Canada). The membrane was blocked with 5% skim powder milk in Tween-Tris buffered saline (TTBS) and incubated in primary antibody overnight. The following primary antibodies were used: VEGF (sc-57496, Santa Cruz, Dallas, TX, USA), PCNA (ab152112, Abcam, Cambridge, MA, USA), αβ-tubulin (#2148, NEB Canada, Whitby, ON, Canada) and β-actin (sc-47778, Santa Cruz). A goat anti-rabbit or anti-mouse IgG-HRP secondary antibody (α-rabbit: 111-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, α-mouse: 115-035-003 Jackson ImmunoResearch Laboratories) incubation was performed, and antibodies were detected with enhanced chemiluminescent reagents [SuperSignal® West Pico Chemiluminescent Substrate (34080, Thermo Fisher Scientific, Mississauga, ON, Canada)]. Densitometry analysis was performed using CARESTREAM MOLECULAR IMAGING SOFTWARE (Molecular Bioimaging, Bend, OR, USA), and signal intensities were normalized to the appropriate loading control from the same membrane.

RNA analysis

RNA was isolated from TA or EDL muscle using the RNeasy Fibrous extraction system (Qiagen Inc.

Canada, Toronto, ON, Canada) and from cultured C2C12 cells using Cells to cDNA lysis buffer (Invitrogen). RNA was reverse transcribed and then quantitative real-time PCR (qPCR) was performed using TaqMan FAM-labelled probes for VEGF-A (Mm00437306_m1; Applied Biosystems Assays on Demand) and Hprt1 (Mm00446968_m1), qPCR Mastermix (4444963; Invitrogen) and the 7500 Fast Real Time PCR system (Invitrogen). VEGF-A mRNA was quantified relative to Hprt1 levels and expressed as $2^{-\Delta Ct}$.

Data analysis

Data are presented as means ± SEM. PRISM 5 (GraphPad Software, La Jolla, CA, USA) was used to analyse data sets using unpaired or paired two-tailed *t*-tests, one-way ANOVA or two-way ANOVA with post hoc tests as appropriate to assess statistical significance ($P < 0.05$).

Results

Prazosin increased capillary number in the skeletal muscle of wild type but not mVEGF$^{-/-}$ mice

Morphometric analysis of untreated male and female WT and mVEGF$^{-/-}$ mice is summarized in Table 1. EDL, TA and gastrocnemius muscle masses relative to body mass were reduced in untreated mVEGF$^{-/-}$ compared to WT mice (Table 1; main effect of genotype; $P < 0.05$). Gender did influence body mass, but did not exert a significant influence on any of the relative muscle mass values. Because of the lack of a detectable gender effect, data from both male and female mice were pooled in subsequent analyses. Morphometric analysis of the major lower hindlimb muscles in untreated and prazosin-treated mice is summarized in Table 2. Prazosin treatment did not influence body mass, relative muscles masses or myofibre cross-sectional areas in WT or mVEGF$^{-/-}$ mice. EDL muscle mass relative to body mass was affected significantly by genotype ($P = 0.04$). Consistent with this observation, we also found that the average myofibre cross-sectional area was smaller in the EDL of mVEGF$^{-/-}$ mice relative to their WT littermates (Table 2). In contrast, the average soleus myofibre cross-sectional area was increased in mVEGF$^{-/-}$ compared to WT mice, although overall relative muscle mass was not altered (Table 2).

We found that the C:F ratio within EDL muscle of mVEGF$^{-/-}$ mice was approximately 50% that of WT mice under control conditions (Fig. 1a,b), consistent with the previous report of reduced gastrocnemius C:F in these mice (Olfert *et al.* 2009). The EDL C:F ratio of WT mice increased significantly after 7 and 14 days of prazosin treatment. However, EDL C:F was not modified by either 7 or 14 days of prazosin in the mVEGF$^{-/-}$ mice (Fig. 1b). We also assessed the influence of mVEGF$^{-/-}$ on capillary network structure within the highly oxidative soleus muscle. Soleus C:F was reduced significantly in mVEGF$^{-/-}$ compared to WT mice, but prazosin treatment did not influence C:F in WT or mVEGF$^{-/-}$ mice (Figure S1a–c).

Consistent with the increase in capillary number, indications of endothelial cell activation (i.e. endothelial cell thickening, luminal protrusions) (Zhou *et al.* 1998, Egginton & Gerritsen 2003) were detectable within skeletal muscle capillaries of 7-day-prazosin-treated WT (Fig. 2a,b) but not mVEGF$^{-/-}$ mice (Fig. 2d,e; Table 3). This analysis was conducted on the EHP muscle, which we considered to have a metabolic and vascular phenotype similar to the EDL muscle (Myrhage & Hudlická 1976, Hansen-Smith *et al.* 1996). Capillaries from prazosin-treated WT mice also were characterized by an increased number of endothelial cells per capillary profile compared to prazosin-treated mVEGF$^{-/-}$ mice (Table 3). Furthermore, some

Table 1 Morphometric comparison of muscles from untreated WT and mVEGF$^{-/-}$ mice

	WT		mVEGF$^{-/-}$	
	Male ($n = 5$)	Female ($n = 5$)	Male ($n = 7$)	Female ($n = 6$)
Body mass (g)*	20.0 ± 0.8	16.3 ± 0.5**	17.7 ± 0.7	18.3 ± 1.3
EDL mass/body mass (mg/g)†	0.43 ± 0.02	0.39 ± 0.02	0.37 ± 0.01	0.36 ± 0.01
Soleus mass/body mass (mg/g)	0.27 ± 0.01	0.26 ± 0.02	0.26 ± 0.02	0.26 ± 0.02
Plantaris mass/body mass (mg/g)	0.69 ± 0.02	0.65 ± 0.02	0.71 ± 0.06	0.61 ± 0.04
Gastrocnemius mass/body mass (mg/g)†	4.32 ± 0.07	4.25 ± 0.08	4.18 ± 0.06	4.04 ± 0.06
TA mass/body mass (mg/g)†	1.62 ± 0.04	1.66 ± 0.04	1.45 ± 0.04	1.46 ± 0.03

All values are represented as average ± SEM.
*Significant interaction between gender and genotype $P = 0.03$.
**Significant difference WT male vs. female, $P < 0.05$, assessed by Bonferroni post hoc test.
†Main effect of genotype; $P < 0.02$.

Table 2 Morphometric comparison of muscles from control and prazosin-treated WT and mVEGF$^{-/-}$ mice

	WT		mVEGF$^{-/-}$	
	Control (n = 10)	Prazosin (n = 4)	Control (n = 12)	Prazosin (n = 6)
Body mass (g)	19.4 ± 09	18.9 ± 0.9	18.7 ± 07	19.0 ± 0.9
EDL mass/body mass (mg/g)†	0.40 ± 0.01	0.41 ± 0.02	0.37 ± 0.01	0.36 ± 0.02
EDL myocyte cross-sectional area (μm^2)†	1570 ± 122	1434 ± 52	1163 ± 49**	1204 ± 33**
Soleus mass/body mass (mg/g)	0.25 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.28 ± 0.01
Soleus myocyte cross-sectional area (μm^2)†	894 ± 87	937 ± 69	1460 ± 155*	1360 ± 169
TA mass/body mass (mg/g)†	1.6 ± 0.03	1.6 ± 0.05	1.4 ± 0.02*	1.4 ± 0.02**
Plantaris mass/body mass (mg/g)	0.64 ± 0.02	0.65 ± 0.02	0.68 ± 0.03	0.66 ± 0.02
Gastrocnemius mass/body mass (mg/g)	4.0 ± 0.08	4.1 ± 0.2	4.1 ± 0.04	4.3 ± 0.05

All values are represented as average ± SEM.
*,**Significant difference relative to corresponding WT, Bonferroni post hoc test; $P < 0.05$, $P < 0.001$ respectively.
†Main effect of genotype; $P < 0.05$.

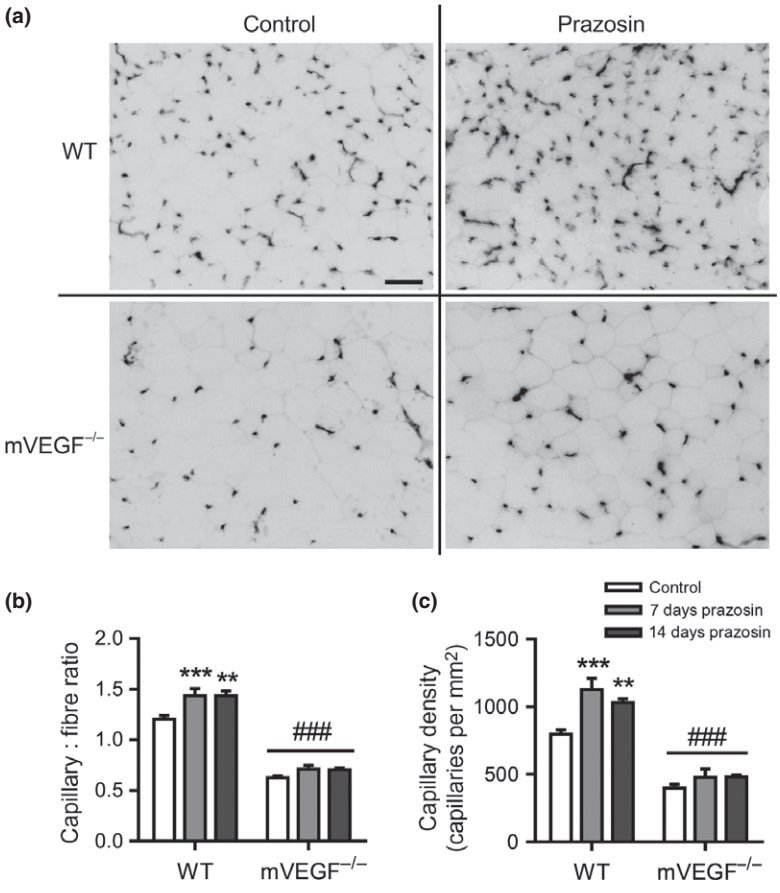

Figure 1 Capillary number in EDL muscle fails to increase in prazosin-treated mVEGF$^{-/-}$ mice. Wild-type (WT) and mVEGF$^{-/-}$ mice were treated with prazosin for 7 or 14 days. (a) Representative images of extensor digitorum longus (EDL) cross sections of 14-day-treated mice, stained with Griffonia simplicifolia lectin-FITC to detect endothelial cells. Fluorescent images were inverted to enhance visualization of the capillaries. Scale bar represents 50 μm. (b,c) Capillary to fibre ratios and capillary densities were calculated and averaged from 3–4 independent fields of view per mouse. For both analyses, two-way ANOVA identified main effects of prazosin treatment ($P = 0.0001$) and of genotype ($P < 0.0001$; n = 4–8 per group). **$P < 0.01$ and ***$P < 0.001$ compared to control WT mice; ###$P < 0.0001$ compared to respective group of WT mice, Bonferroni post hoc analysis.

capillaries in the mVEGF$^{-/-}$ mice showed indications of endothelial cell damage and/or capillary regression (i.e. membrane blebbing; the absence of cytosolic structures) after 14 days of prazosin treatment, while these features were not detectable in capillaries of WT mice (Fig. 2c,f). No significant influence of genotype or prazosin treatment was detectable by Western blot analysis of PCNA protein levels within TA muscle extracts (Figure S2).

Arterial adaptations to increased blood flow are impaired in mVEGF$^{-/-}$ mice

Prazosin also has been reported to increase arteriole size and number in rat gracilis muscle (Price & Skalak 1996). Therefore, we assessed the frequency and size of α-smooth muscle actin-positive (SMA+) vessels as an indication of possible arteriolarization of pre-existing capillaries and/or remodelling of pre-existing arte-

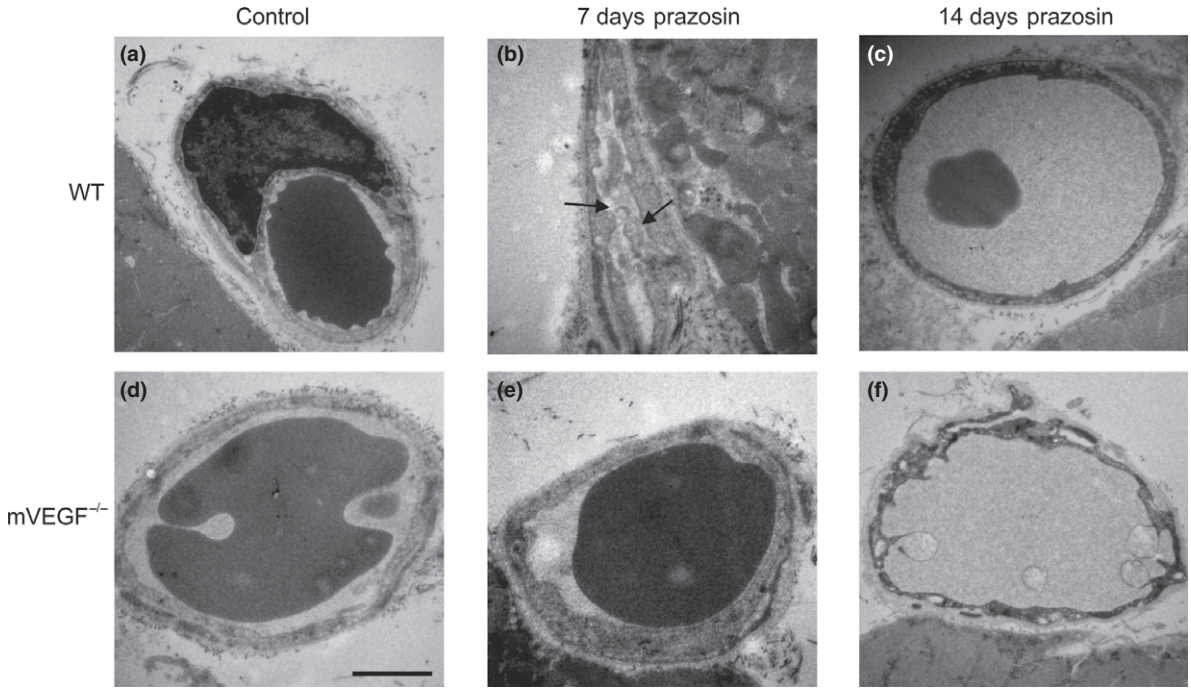

Figure 2 Ultrastructural differences between capillaries of WT and mVEGF$^{-/-}$ mice following prazosin treatment. Representative electron microscope images (20 000× magnification) are shown of capillaries within the EHP muscle of control and prazosin-treated mice. Scale bar represents 1 μm. (a, d) No overt structural differences were observed in control mVEGF$^{-/-}$ mice compared to their WT littermates. (b, e) Following 7 days of prazosin treatment, capillaries in WT mice showed thickening of the capillary wall, ruffling of the membrane and luminal projections (denoted by arrows), but these alterations were not evident within the capillaries of mVEGF$^{-/-}$ mice. (c, f) After 14 days of prazosin treatment, indications of endothelial cell damage and capillary regression were observed in capillaries of mVEGF$^{-/-}$ mice. These morphological features were absent in the capillaries of 14-day-treated WT mice.

Table 3 Morphological features of capillaries within EHP muscle

	WT		mVEGF$^{-/-}$	
	Control (n = 13)	Prazosin (n = 17)	Control (n = 6)	Prazosin (n = 15)
Endothelial Cells/Capillary†	1.3 ± 0.1	1.7 ± 0.2*	1.3 ± 0.2	1.1 ± 0.1
Luminal Protrusions /Capillary†,‡	0.46 ± 0.1	1.1 ± 0.3	0 ± 0.0	0.5 ± 0.2
Pericyte Associations/Capillary	1.3 ± 0.2	1.1 ± 0.2	1.7 ± 0.2	1.3 ± 0.1
Presence of Vacuoles	+	++	+	+
Thickened Endothelial Cell	–	+	–	–

*Significant difference relative to control WT and prazosin mVEGF$^{-/-}$, Bonferroni post hoc test; $P < 0.05$, $P < 0.01$ respectively.
†Main effect of genotype; $P < 0.05$.
‡Main effect of prazosin treatment; $P < 0.05$.

rioles in response to prazosin treatment (Fig. 3). A significant genotype effect was observed for both SMA+ vessel density and size in EDL muscle ($P = 0.002$ and $P = 0.03$ respectively) (Fig. 3b,c). Despite reductions in the density of both capillaries and SMA+ vessels, the capillaries/SMA+ vessel ratio was not maintained in mVEGF$^{-/-}$ mice (65 ± 5 in WT vs. 32 ± 4 in mVEGF$^{-/-}$; $P < 0.05$, $n = 6$). The average density of SMA+ vessels (per mm^2) increased moderately after 14 days of prazosin treatment in the WT mice ($P < 0.01$ vs. control). In contrast, prazosin treatment did not affect the SMA+ vessel density in the EDL of mVEGF$^{-/-}$ mice, resulting in a significant difference between 14-day-prazosin-treated WT and mVEGF$^{-/-}$ mice ($P < 0.001$; Fig. 3b). The average perimeter of SMA+ vessels was not affected by prazosin in WT mice, while 14 days of prazosin significantly reduced the perimeter of SMA+ vessels in mVEGF$^{-/-}$ EDL compared to control ($P < 0.05$; Fig. 3c). Likewise, SMA+ vessels in the EDL of mVEGF$^{-/-}$ mice were significantly

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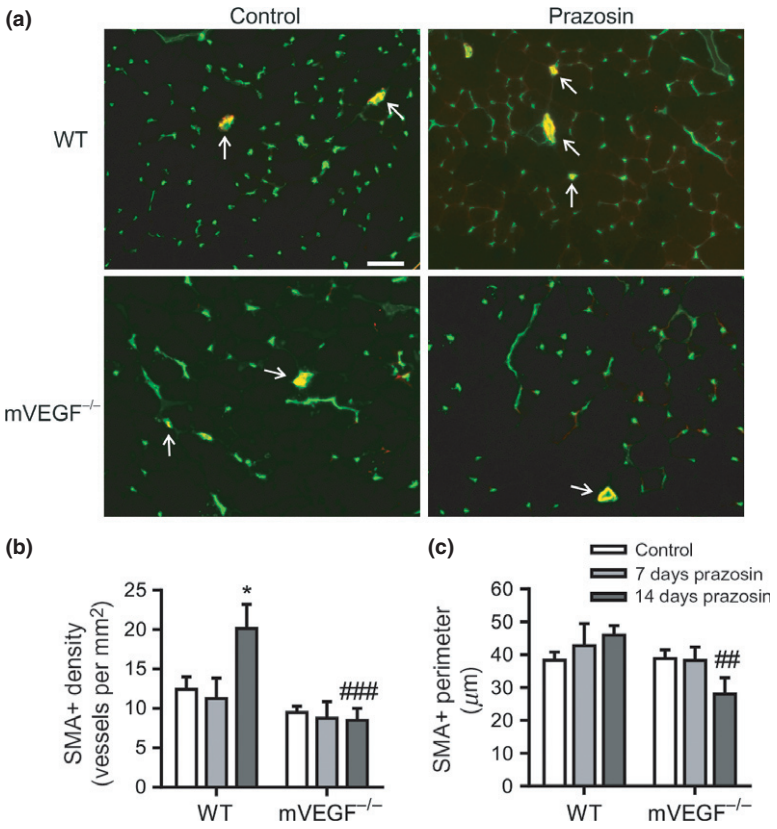

Figure 3 Arteriolar remodelling is impaired in the EDL of mVEGF$^{-/-}$ mice in response to prazosin treatment. (a) Representative images of EDL cross-sections from control and 14-day-prazosin-treated WT or mVEGF$^{-/-}$ mice, co-stained with anti-smooth muscle actin-Cy3 (red) and Griffonia simplificolia lectin-FITC (green). Arrows point to SMA+ vessels. Scale bar represents 50 μm. (b) The average number of SMA+ vessels per mm^2 was calculated based on analysis of 3–4 independent fields of view per mouse. Two-way ANOVA indicated a significant genotype effect ($P = 0.002$) and a significant interaction between genotype and treatment ($P = 0.03$). *$P < 0.05$ compared to WT control; ###$P < 0.001$ compared to 14-day-prazosin-treated WT mice, Bonferroni post hoc analysis. (c) The effect of prazosin treatment and mVEGF deletion on SMA+ vessel size was assessed by comparing the average perimeter of SMA+ vessels. A significant genotype effect was observed ($P = 0.03$), as well as a significant interaction between genotype and prazosin treatment ($P = 0.02$), by two-way ANOVA analysis. ##$P < 0.01$ compared to 14-day-treated WT mice, Bonferroni post hoc analysis. $n = 4$–8 mice per group for each of the above analyses.

smaller than their WT counterparts after 14 days of prazosin ($P < 0.01$; Fig. 3c). In the soleus muscle, SMA+ vessel density was reduced in mVEGF$^{-/-}$ compared to WT mice, and there was no influence of prazosin treatment (Figure S1d). The average size of SMA+ vessels was lower in 7-day-prazosin-treated mVEGF$^{-/-}$ animals compared to their WT counterparts (Figure S1e).

Nitric oxide stimulates VEGF production in muscle cells

Previous studies have reported that prazosin treatment increases VEGF-A within skeletal muscle, although it has been postulated that this increased expression occurs predominantly within the endothelial compartment (Milkiewicz *et al.* 2001, Rivilis *et al.* 2002). Consistent with these studies, we observed a substantial increase in VEGF-A mRNA in TA muscle of WT mice treated with prazosin for 7 days (Fig. 4a). As expected, VEGF-A mRNA was reduced severely in the muscle of mVEGF$^{-/-}$ compared to WT mice. However, prazosin treatment failed to increase VEGF-A mRNA in these muscles (Fig. 4a). The induction of VEGF-A mRNA is unlikely to be a direct effect of prazosin on skeletal myocytes, as we found that overnight incubation of C2C12 myocytes with prazosin (0.1 or 1 μM) did not modulate VEGF-A mRNA levels (Fig. 4b).

In considering a potential mechanism by which myocyte-derived VEGF-A may be altered in response to increased blood flow, we hypothesized that shear stress-induced secretion of an endothelial-derived factor induces the increased VEGF-A production in muscle. We postulated that nitric oxide (NO) may control skeletal myocyte VEGF-A production, as it is well established that endothelial cell production of this gaseous signalling molecule increases in response to shear

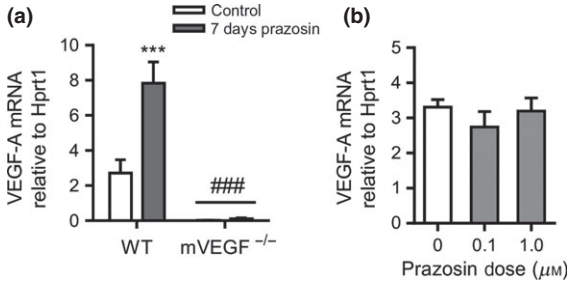

Figure 4 Prazosin increases VEGF-A mRNA in the muscle of WT but not VEGF$^{-/-}$ mice. (a) VEGF-A mRNA in TA muscle from control and 7-day-prazosin-treated WT and mVEGF$^{-/-}$ mice was assessed by qPCR. Values were normalized to the housekeeping gene HPRT1 and expressed as $2^{-\Delta Ct}$. Significant treatment ($P < 0.001$) and genotype ($P < 0.001$) effects were detected by 2-way ANOVA; $n = 4$. ***$P < 0.001$ vs. control; ###$P < 0.001$ vs. treatment-matched WT group, Bonferroni post hoc analysis. (b) C2C12 myocytes were incubated overnight with prazosin (0.1 or 1.0 μM). VEGF-A mRNA was analysed by qPCR, and expressed relative to Hprt1 mRNA as $2^{-\Delta Ct}$. No significant treatment effect was detected ($P > 0.05$) by one-way ANOVA; $n = 3$.

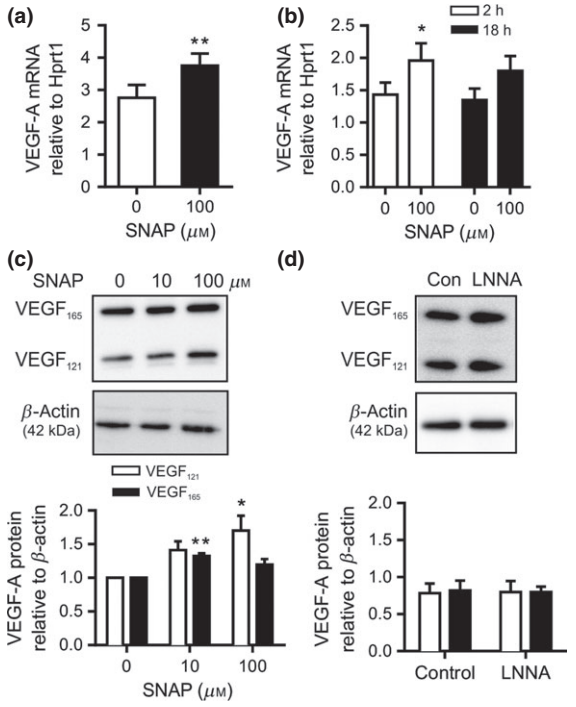

Figure 5 Nitric oxide stimulates VEGF-A production in EDL muscle and in cultured muscle cells. (a) Paired EDL muscles from WT mice were incubated for 2 h *ex vivo* in the presence or absence of the NO donor SNAP (100 μM). Whole muscle VEGF-A mRNA was analysed by qPCR and expressed relative to Hprt1 mRNA as $2^{-\Delta Ct}$. **$P < 0.01$ vs untreated, assessed by paired *t*-test; $n = 4$. (b) C2C12 myoblasts were treated with SNAP (100 μM) for 2 or 18 h. VEGF-A mRNA was analysed by qPCR and expressed relative to Hprt1 mRNA as $2^{-\Delta Ct}$. *$P = 0.04$ vs. untreated, paired t-test; $n = 4$. (c) VEGF-A_{121} and VEGF-A_{165} protein levels in cell lysates were measured by Western blot and normalized to β-actin. SNAP treatment significantly increased both VEGF-A_{121} and VEGF-A_{165} levels ($P = 0.02$ and $P = 0.006$, respectively, one-way ANOVA). *$P < 0.05$ and **$P < 0.01$ vs. respective controls, Tukey post hoc analysis; $n = 4$ independent experiments per group. (d) C2C12 myoblasts were treated overnight with a competitive inhibitor of NO synthase, LNNA (30 μM). VEGF-A_{121} and VEGF-A_{165} protein levels were measured by Western blot and normalized to β-actin. LNNA treatment had no significant effect on basal levels of VEGF-A proteins ($P > 0.05$; $n = 4$ independent experiments per group).

stress (Buga *et al.* 1991, Fleming & Busse 1999) and that it plays a role in shear stress-induced angiogenesis (Baum *et al.* 2004). Two hour *ex vivo* incubation of EDL muscles from WT mice with the NO donor SNAP induced a significant increase in muscle VEGF-A mRNA levels (Fig. 5a). In cultured C2C12 cells, two hour treatment with SNAP increased VEGF-A mRNA ($P = 0.04$; $n = 4$); a similar but non-significant trend was observed after 18 h treatment ($P = 0.11$; $n = 3$) (Fig. 5b). SNAP treatment also elevated levels of cellular VEGF-A protein (Fig. 5c). Considering the potential for endogenous NO produced by skeletal myocyte nNOS to contribute to basal production of VEGF-A in myocytes, we examined VEGF-A levels in C2C12 cells treated overnight with the NOS inhibitor LNNA. However, LNNA did not modulate VEGF-A protein level (Fig. 5d).

Discussion

We demonstrate here that muscle-derived VEGF-A is a critical regulator of microvascular adaptations to increased blood flow in skeletal muscle. There was no increase in EDL C:F ratio in mVEGF$^{-/-}$ mice in response to prazosin treatment. Furthermore, the number and size of SMA+ vessels was significantly lower in the EDL of prazosin-treated mVEGF$^{-/-}$ mice when compared to their WT littermates. We also provide evidence that myocyte VEGF is upregulated by NO, indicating that NO may serve a key paracrine function in facilitating crosstalk from the endothelium to the skeletal myocytes.

It has been well established that increased blood flow provokes remodelling of the microvascular network within skeletal muscle. Chronic administration of the α-adrenergic inhibitor prazosin produces sustained increases in blood flow and capillary shear stress within glycolytic muscles, resulting in expansion of the capillary network (Fulgenzi *et al.* 1998, Zhou *et al.* 1998, Milkiewicz *et al.* 2001, Rivilis *et al.* 2002). Consistent with the previous studies, we observed that chronic increases in shear stress

achieved by prazosin treatment increased the C:F ratio in the EDL of WT animals after 7 and 14 days.

A longstanding premise is that flow-induced angiogenesis is an autonomous endothelial cell process that is promoted by the activation of shear stress-dependent signal pathways and that occurs independently of the status of the parenchymal tissue (Egginton 2011a, b). Based on experiments utilizing VEGF-trap (Williams *et al.* 2006a), VEGF-A is appreciated to play a critical role in this process. Evidence that an increase in shear stress can stimulate endothelial cell production of VEGF-A (Milkiewicz *et al.* 2001, Gee *et al.* 2010, dela Paz *et al.* 2012) provided additional support for the concept of an autocrine role of VEGF-A in flow-induced angiogenesis. However, the majority of VEGF-A present within skeletal muscle is thought to originate from skeletal myocytes rather than endothelial cells (Delavar *et al.* 2014, Hoier & Hellsten 2014). VEGF-A 165 and 189 are the predominant isoforms present within skeletal muscle (Gustafsson *et al.* 2005) and these isoforms bind with high affinity to heparan sulphate proteoglycans within the extracellular matrix (Park *et al.* 1993). Matrix-bound forms of VEGF-A may be released by enzymes such as matrix metalloproteinases and plasmin, resulting in the activation of endothelial cells and the initiation of capillary sprouting (Lee *et al.* 2005). A robust body of evidence indicates that increased production of myocyte-derived VEGF-A in response to a metabolic challenge is essential for the subsequent process of angiogenesis (Olfert *et al.* 2010, Delavar *et al.* 2014, Gorman *et al.* 2014), consistent with a close link between tissue metabolism and signals that regulate capillary growth. Because shear stress-induced angiogenesis is postulated to occur independently of altered tissue metabolism, we hypothesized that it would not rely on the presence of myocyte-derived VEGF-A.

By utilizing mice deficient for skeletal muscle VEGF-A (which retain expression of endothelial-derived VEGF-A), our study instead demonstrates that myocyte-derived VEGF-A is required for an appropriate increase in C:F in glycolytic muscle in response to sustained vasodilator treatment. By electron microscopy, capillaries in the EHP muscle of WT, but not mVEGF$^{-/-}$, mice showed indications of endothelial cell activation and the formation of luminal protrusions in response to prazosin. Proliferation during the process of luminal splitting angiogenesis is known to be modest (Zhou *et al.* 1998). We did observe an increased number of endothelial cells per capillary profile in prazosin-treated WT, which was not detected in the mVEGF$^{-/-}$, mice, suggesting a reduced capacity for endothelial cell proliferation in mVEGF$^{-/-}$ mice. To date, very little is known about the signalling pathways that contribute to shear stress-induced capillary growth; thus, the specific cellular events that are disrupted in the absence of myocyte-derived VEGF-A remain to be established.

The mouse model used in the current study is characterized by the skeletal and cardiac myocyte deletion of VEGF-A during the late embryogenesis to early post-natal period (Olfert *et al.* 2009). A limitation of this model is that mouse phenotype is influenced by the role of VEGF-A in the early development of the skeletal muscle microcirculation. Consistent with previous reports of reduced muscle C:F in the mVEGF$^{-/-}$ mice (Olfert *et al.* 2009, Gorman *et al.* 2014), we detected reduced C:F in both the glycolytic EDL and the oxidative soleus muscle of untreated mVEGF$^{-/-}$ mice, irrespective of gender. In contrast to loss of skeletal muscle VEGF during embryogenesis, the inducible deletion of VEGF-A in skeletal muscle of adult mice does not reduce capillary number (Delavar *et al.* 2014). Together, these findings indicate that myocyte-derived VEGF-A plays a uniquely critical role in establishing and/or stabilizing the nascent capillary networks within skeletal muscle during late embryogenesis and suggests that myocyte VEGF-A is not needed to maintain capillaries within a mature capillary network. This is corroborated further by data indicating that autocrine VEGF-A conveys critical endothelial cell survival signals (Lee *et al.* 2007).

Prazosin treatment has been shown to stimulate the development of new terminal and arcade arterioles within the skeletal muscle microcirculation (Price & Skalak 1996). We observed that 14 days of prazosin treatment resulted in an increase in the number of SMA+ microvessels within the EDL muscles of wild type mice. We did not observe a significant alteration in the number or size of SMA+ microvessels within the EDL muscle of the mVEGF$^{-/-}$ mice under basal conditions. However, flow-induced remodelling of SMA+ vessels was deficient in the EDL of mVEGF$^{-/-}$ mice, as both the number and size of SMA+ vessels was significantly lower in 14-day-prazosin-treated mVEGF$^{-/-}$ mice compared to their respective prazosin-treated wild type littermates. Interestingly, we found that the basal number of SMA+ vessels was reduced in the soleus muscle of mVEGF$^{-/-}$ mice, although this was not affected further by prazosin treatment. Together, these data suggest that myocyte-derived VEGF-A is not an absolute requirement for the formation of SMA+ vessels, but that it plays a significant role in facilitating the appropriate flow-induced remodelling of vessel number and size.

In contrast, Delavar and colleagues reported an increase in the number and size of SMA+ vessels following the inducible deletion of VEGF-A in adult skeletal muscle (Delavar *et al.* 2014), which would suggest an unusual growth-suppressive function of

VEGF-A within these microvessels. Our data are consistent with several other studies showing the positive involvement of VEGF-A in arterial remodelling. A global reduction of VEGF-A levels resulted in a reduced number of collateral arteries during development of the mouse brain (Lucitti *et al.* 2012). Adult mice that under-express VEGF-A were shown to have reduced numbers of collateral arteries in skeletal muscle, correlating functionally with a blunted recovery of hindlimb blood flow following femoral artery ligation, while the opposite was observed in mice overexpressing VEGF-A (Clayton *et al.* 2008). Due to the global nature of the genetic manipulations, these studies could not discriminate the involvement of specific cellular sources of VEGF-A. Currently, it is not known whether the role of VEGF-A in arteriolar remodelling is direct (ie. via receptor dependent signalling) or indirect, as a consequence of lack of alteration in network conductance due to the failure of capillary network expansion (Pries *et al.* 2010).

Prazosin did not influence C:F or SMA+ remodelling in the soleus muscle. The soleus muscle in rats was characterized previously to have a high basal blood flow, which was not increased by α-adrenergic receptor blockade using phentolamine (Laughlin & Armstrong 1982, 1987). While the same analyses remain to be conducted in mouse soleus muscle, it is likely that the lack of vascular remodelling we observed is associated with a minimal influence of prazosin on soleus blood flow.

Interestingly, we found a significant reduction in the average skeletal myocyte cross-sectional area in the EDL of mVEGF$^{-/-}$ mice. A previous study did not detect a difference in myocyte cross-sectional area in the same mice (Olfert *et al.* 2009). The difference in findings may arise because the earlier study assessed the gastrocnemius muscle, which has a broader range of fibre types compared to the EDL muscle (Augusto *et al.* 2004). Our finding raises the intriguing possibility that myocyte-derived VEGF-A acts as a myotrophic factor in the highly glycolytic EDL muscle. There is support for a direct cellular action of VEGF-A on myocyte hypertrophy and differentiation (Germani *et al.* 2003, Bryan *et al.* 2008). However, it also is possible that myocyte hypertrophy in the EDL is limited as a consequence of insufficient muscle capillarization. In contrast, we observed a modest increase in myocyte cross-sectional area within the soleus muscle. It is possible that this change in size coincides with the adaptation of this oxidative muscle to a more glycolytic phenotype as a consequence of poor vascularization.

Our findings provide novel evidence that the skeletal muscle angiogenesis in response to a flow stimulus requires crosstalk between endothelial cells and skeletal myocytes. Our data support the hypothesis that NO modulates flow-induced angiogenesis by acting as a paracrine signal that links alterations in blood flow to the production of VEGF-A within myocytes. This concept first was suggested by experiments in which infusion of the NO donor sodium nitroprusside caused significant increases in VEGF-A mRNA in rat skeletal muscle in the absence of an exercise stimulus (Benoit *et al.* 1999). Conversely, inhibition of NO production via infusion of L-NAME significantly attenuated the acute exercise-induced increase in VEGF-A mRNA in rat skeletal muscle (Gavin *et al.* 2000). However, interpretation of those experiments was confounded by the simultaneous influence of these treatments on mean arterial pressure and local muscle blood flow. We provide evidence that exogenous NO, acting independent of alterations in hemodynamics, stimulates VEGF-A production in EDL muscle *ex vivo* and in cultured myoblasts. Our data, which illustrate the capacity of NO to modulate muscle VEGF-A production, strongly suggest, but do not exclusively prove, that flow-dependent NO synthesis by endothelium mediates VEGF upregulation in the adjacent muscle fibres. This concept fits well with evidence in the literature that NO regulates vascular remodelling. For example, inhibition of NO activity prevented capillary growth in response to muscle activity (Hudlická *et al.* 2000), and deletion of endothelial NO synthase (NOS) prevented prazosin-induced angiogenesis (Williams *et al.* 2006b). Inhibition of NO activity also was shown to prevent ischaemia-driven collateral vessel formation within the coronary circulation (Matsunaga *et al.* 2000).

In summary, we show for the first time that myocyte-derived VEGF-A is an essential regulator of microvascular adaptations to increased blood flow within both capillaries and SMA+ microvessels of glycolytic muscle. Our results demonstrate that the production of VEGF-A by other cell types (i.e. endothelial cells) cannot compensate for the loss of the myocyte source of VEGF-A. We propose a signal loop in which flow-stimulated endothelial NO synthesis modulates skeletal myocyte production of the potent angiogenic factor VEGF-A, in turn promoting flow-mediated vascular remodelling. Thus, our data highlight the importance of cellular cross-talk within the local microenvironment in the maintenance of appropriate microvascular remodelling responses.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Supporting Information

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

Figure S1. Soleus muscle microvasculature in WT and mVEGF$^{-/-}$ mice.

Figure S2. PCNA protein level in TA muscle.

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