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Shear stress-induced angiogenesis in mouse muscle is independent of the vasodilator mechanism and quickly reversible

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6 figures, 1 supplementary figure, 1 supplementary table

Abstract

Aim: Is modulation of skeletal muscle capillary supply by altering blood flow due to a presumptive shear stress response *per se*, or dependent on the vasodilator mechanism?

Methods: The response to four different vasodilators, and co-treatment with blockers of NO and prostaglandin synthesis, were compared. Femoral artery blood flow was correlated with capillary to fibre ratio (C:F), and protein levels of putative angiogenic compounds.

Results: All vasodilators induced a similar increase in blood flow after 14 days, with a similar effect on C:F (1.62±0.05, 1.60±0.01, 1.57±0.06, 1.57±0.07, respectively, all $P<0.05$ *vs*. control 1.20±0.01). Concomitant inhibitors revealed differential effects on blood flow and angiogenesis, demonstrating that a similar response may have different signalling origins. The time course of this response with the most commonly used vasodilator, prazosin, showed blood flow increased from 0.40 $ml.min^{-1}$ to 0.61 $ml.min^{-1}$ by 28 days ($P<0.05$), dropped within 1 week after cessation of treatment (0.54 $ml.min^{-1}$; $P<0.05$) and returned to control levels by 6 weeks. In parallel with FBF capillary rarefaction began within 1 week ($P<0.05$), giving C:F values similar to control by 2 weeks. Of the dominant signalling pathways, prazosin decreased muscle VEGF but increased its cognate receptor Flk-1 (both $P<0.01$), levels of eNOS varied with blood flow ($P<0.05$), Ang-1 initially increased while its receptor Tie-2 was unchanged, with only modest changes in the anti-angiogenic factor TSP-1.

Conclusion: Hyperaemia-induced angiogenesis, likely in response to elevated shear stress, is independent of the vasodilator involved, with a rapid induction and quick regression following stimulus withdrawal.

Introduction

Angiogenesis is the postnatal mechanism whereby an existing microvascular network is expanded in response to a changing metabolic and mechanical environment (Hudlická et al., 1992). For example, endurance exercise increases haemodynamic forces within skeletal muscle microcirculation accompanying the functional hyperaemia and deformation of blood vessels during a duty cycle (Egginton, 2009). Vasoactive metabolites mediate arteriolar vasodilatation, and the accompanying increase in vascular conductance is a major stimulus for exercise-induced angiogenesis (Prior et al., 2004). As capillaries have low compliance, elevated microvascular perfusion leads to an elevated capillary shear stress, as confirmed by intravital microscopy observations (Hudlická et al., 1992). Canonical, sprouting angiogenesis is invoked by increased muscle activity (Hudlická et al., 1992, Hansen-Smith et al., 1996). However, physiological angiogenesis may also occur through capillary splitting, intussusception, and elongation, in response to a number of different stimuli (Egginton et al., 2001).

Orally administered vasodilators may expand the microvascular bed (Hudlická, 1998), and chronic treatment stimulates a specific form of angiogenesis termed longitudinal splitting (Egginton et al., 2001). Muscle capillary neoformation occurs in rats treated with dipyridamole (Tornling et al., 1980), and in rabbits treated with adenosine or with propentofylline (Ziada et al., 1984), but the α_1-adrenoreceptor antagonist prazosin offers a targetted increase in capillarisation of skeletal muscle due to the differential adrenoreceptor receptor density among tissues (Hudlická, 1998) (Baum et al., 2004). Other vasoactive drugs have been less effective, likely due to second order effects on either cardiac or skeletal muscle. For example, treatment with nitroglycerine (an NO donor), diltiazem (a calcium channel blocker), or propranolol (a non-selective beta-blocker) did not affect the capillary to fibre ratio (C:F) in rat

EDL or soleus (Torres et al., 1994). An important question is, therefore, whether the mechanism of vasodilatation affects the extent of shear stress-induced angiogenesis.

During development and adaptive remodelling in adult organs, newly grown blood vessels are often pruned to optimise functional capacity of the microcirculation, a process known as angioadaptation (Zakrzewicz et al., 2002). Vascular remodelling after withdrawal of a specific angiogenic stimulus has not been widely studied, although a decrease in capillary supply may follow reduced muscle activity, e.g. a 6.3% reduction in capillary density in long-distance runners only two weeks after stopping training (Houston et al., 1979), and more recently in exercise trained mice where a complex pattern of pro- and anti-angiogenic factor changes was observed (Olenich et al., 2014). The speed of vascular regression is important as it may influence progression of impaired function associated with e.g. bed rest and use of angiotherapies. Existing animal models of vessel regression (e.g. muscle denervation, limb suspension, tenotomy) all have limitations when determining the speed of regression. For example, studies in skeletal muscle with disuse atrophy following hindlimb unloading demonstrate a decrease in blood flow (McDonald et al., 1992), capillary luminal diameter and C:F (Kano et al., 2000), despite an increase in capillary density (Tyml et al., 1999), probably because the rate of muscle atrophy was greater than that of capillary regression. While some studies have explored changes in gene expression and protein levels during shear stress-induced angiogenesis (Wragg et al., 2014), little attention has focused on the physiological response to cessation of vasodilator treatment and subsequent capillary regression. Here, we explore the effects of chronic increase in blood flow, and examined how quickly the microcirculation reverts to pre-treatment levels once the angiogenic stimulus is removed.

Studies examining the molecular pathways involved in endothelial mechanotransduction of haemodynamic stimuli have largely focused on VEGF and NO, as these regulate shear-mediated angiogenesis in a spatiotemporal manner (Egginton, 2011). VEGF is vital in physiological angiogenesis: its blockade results in the absence of capillary growth following chronic hyperaemia or muscle overload (Williams et al., 2006a, Williams et al., 2006c, Uchida et al., 2015). VEGF and its receptor VEGFR2 are upregulated by elevated shear stress, with peak expression preceding the increase in capillarisation (Milkiewicz et al., 2006). Pharmacological blockade of NOS isoforms, or genetic ablation of eNOS but not nNOS, also led to suppression of prazosin-induced angiogenesis (Williams et al., 2006a). Increased shear stress associated with tissue hyperaemia leads to release of NO and prostaglandins, in particular prostacyclin (PGI_2). Blockade of prostaglandin synthesis by indomethacin likewise attenuates angiogenesis that accompanies increased muscle activity due to electrical stimulation (Pearce et al., 2000). It is therefore unclear whether different forms of vasodilatation are differentially sensitive to blockade of NO or prostaglandin production.

We tested the hypothesis that the mechanism of vasodilatation does not determine the extent of shear stress-induced angiogenesis, and vasodilators have no direct angiogenic effects *in vivo* independent of blood flow. To achieve this, we used four different vasodilators, and inhibited NO and prostacyclin production, two archetypal downstream mediators of vascular tone and blood flow. Given the low mitotic activity associated with this form of angiogenesis (Egginton, 2011), we reasoned that the time course of microvascular growth and regression after vasodilator treatment was stopped would be short. Finally, we determined expression levels of some key regulatory factors to establish potential signalling pathways. The findings may be important in developing effective short-acting angiotherapies.

Materials and Methods

Animals

All work was performed in accordance with the UK Animal (Scientific Procedures) Act 1986. The procuration of animals, the husbandry and the experiments conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Male C57/BL10 mice at least 6 wk old (from Harlem or Charles River, UK; body mass 23.5±0.4g, n=4-6 per group) were housed at 21˚C with a 12:12 light:dark cycle. Males were chosen in order to avoid confounding influences on vasculature remodelling accompanying the estrus cycle. All animals were kept in an enriched environment and given standard laboratory feed and water *ad libitum.*

Administration of different vasoactive compounds

All vasodilator drugs were given in drinking water for a period of 14 d: prazosin (α1 adrenoreceptor antagonist, 50mg.l^{-1}), verapamil (L-type calcium channel inhibitor, 35mg.l^{-1}), cromakalim (K_{ATP} channel opener, 1mg.l^{-1}), and ethanol (impairs actin-myosin interaction and decreases cytosolic–free Ca^{2+} in arteriolar smooth muscle, 3%; (Zhang et al., 1992)). Potential haemodynamic effects of vasodilators were minimised by choosing doses based on previous literature, and pilot studies. The lack of any significant change in blood pressure or heart rate suggests regional dilatation was compensated elsewhere in the cardiovascular system, so the effects on e.g. cardiac output are likely to be small. The non-specific nitric oxide synthase (NOS) inhibitor N_w-nitro-L-arginine (L-NNA, 100mg.l^{-1}) or non-selective inhibitor of cyclooxygenase 1 and 2, indomethacin (50mg.l^{-1}) were used in each of the vasodilator

groups to elucidate the possible roles that NO and prostaglandin may play in shear stress-induced angiogenesis, respectively. Inhibitor treatment was concurrent with vasodilator treatment. Previous studies from our group using individual vasodilators in rats (all experiments exploring the mechanisms of angiogenesis have yielded closely similar results in rats and mice) showed no significant effects of these inhibitors alone (Pearce et al., 2000, Williams et al., 2006a), as expected given the low basal NO and prostaglandin expression.

Time course of vasodilator response

In previous studies (Williams et al., 2006a) a steady state capillarisation was reached by 4 wk prazosin treatment. Mice were therefore given vasodilators in drinking water and sampled at 2, 4, 7, 14 and 28 d, after which treatment was ended and the mice were given access to untreated drinking water. Groups of mice were then sampled 3, 7, 14, 28 and 42 d after cessation of treatment.

Systemic effects of vasodilator treatment

Mice were anaesthetised i.p. with ketamine (0.1 mg/kg, Pharmacia) and xylazine (0.01 mg/kg, Millpledge Pharmaceuticals, UK), with core temperature controlled by a heating pad. Arterial blood pressure (ABP) and heart rate were measured as described previously (Williams et al., 2006a). Briefly, the right carotid artery was cannulated (PP10) for ABP measurements, and the trachea cannulated (PP50) to aid spontaneous ventilation. A perivascular flow probe (Transonic 0.5VB, Linton Instrumentation, Norfolk, UK) on the upper portion of the femoral artery, adjusted to minimise strain, was used to record hindlimb blood flow. Previous studies have demonstrated a clear relationship between hindlimb blood flow and individual muscle blood flow in rats (Hargreaves et al., 1990, Hudlická and Brown, 2009). All recordings were made with

PowerLab (AD Instruments, Oxford) and LabChart software. The procedure took <1h to minimise dehydration or the need for further anaesthesia.

Histochemistry

Animals were killed by cervical dislocation before the *m. extensor digitorum longus* (EDL; a mixed fast muscle) was dissected and snap-frozen in liquid nitrogen-cooled isopentane. 10µm cryosections were prepared and allowed to air-dry at room temperature (RT). Capillary staining was performed at RT for 30 min on cool acetone-fixed sections using rhodamine-conjugated *Griffonia simplicifolia* lectin-1 (Vector; 1:200). Cell proliferation was measured using proliferating cell nuclear antigen (PCNA, Santa Cruz; 1:100,). Sections were incubated with secondary antibody (1:50 CY2 conjugated donkey anti-rabbit, Jackson, and 1:100 rhodamine conjugated GSL-1 lectin, Vector) to identify sites of capillary-associated cell proliferation. Sections were rinsed and mounted in glycerol. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining to measure cell apoptosis was carried out following manufacturer's instructions (Invitrogen). Briefly, Proteinase K solution was applied to air-dried sections, and following wash and quenching, transferred into TdT reaction mix. Slides were incubated at 37°C and developed using streptavidin-HRP detection developed with DAB solution, counterstained with methyl green, cleared in xylene and mounted in Histomount (Invitrogen). Sections were viewed under fluorescent illumination (Zeiss Axioskop 2 microscope) using proprietary software (Axiovision, Zeiss), and images captured on an MRc digital camera. Capillaries and fibres were counted as previously described (Egginton, 1990a, Egginton, 1990b). Briefly, four non-overlapping images were taken per section, each in the same relative position and equally spaced, and a square lattice counting frame (area 0.194 mm^2) superimposed at a total magnification of x250.

Although capillary supply to the muscle may be expressed as either capillary density (mm^{-2}) or as capillary to fibre ratio (C:F), the latter is less sensitive to modest inter-animal variability in fibre size (Egginton, 1990a), and was therefore used throughout as an index of angiogenic activity. It was not logistically possible to include time-controls for all points, but we have previously conducted such controls for up to 14 day treatment (Williams et al., 2006a) with no significant change in C:F.

Biochemical analyses

SDS-PAGE was used for electrophoretic analysis of proteins, following standard western blotting techniques. Briefly, samples were snap-frozen and crushed in liquid nitrogen, and homogenised in RIPA lysis buffer with added protease inhibitors (1mM P.I.C; 1mM PMSF; 1mM Na_3Vo_4; Sigma) for extraction. Protein concentration was estimated using bovine serum albumin as a standard. Samples were run simultaneously with equal protein loading (100µg/lane) at 4°C (150V, 1h; BioRad), transferred onto polyvinylidene membranes (100V, 1-2h; BioRad) on ice. Non-specific protein binding was blocked with 1% milk and probed with primary antibodies (overnight at 4°C), then incubated with secondary antibodies (typically 1:5,000 for 1h at RT; Jackson goat-anti mouse cat#115-085-166, goat-anti rabbit cat#111-035-144) and visualised by enhanced chemiluminscence (ECL Femto; Pierce). The following primary antibodies were used: eNOS (1:200, BD Transduction Laboratories, cat#610293), VEGF (1:500, Santa Cruz Biotechnology, cat#SC-57496), Flk-1 (1:500; Santa Cruz Biotechnology, cat#SC-504), Ang-1 (1:500, Rockland, cat#100-401-403), Tie-2 (1:1000, R&D Systems, cat#AF762), TSP-1 (1:1000, R&D Systems, cat#AF3074). All other materials were from Sigma Aldrich, UK, unless otherwise stated. ImageJ software (NIH) was used to quantify relative protein levels from densitometric analysis of photosensitive film (Amersham). Total protein was

quantified (MemCode Reversible Stain; Pierce), and successful protein transfer confirmed using Coomassie stain of membranes (Pierce).

Statistical analysis

All data are presented as mean ± S.E.M. Statistical significance between groups was established using ANOVA with Fishers' PLSD post hoc test using a 5% significance level.

Results

Systemic dilator response

Heart rate and blood pressure remain unaltered during drug treatment, despite increases in hindlimb blood flow as a consequence of local vasodilatation (Supplementary Table 1), suggesting that the reduced peripheral resistance was adequately compensated by an increased cardiac output (i.e. an effective baroreceptor reflex). There was some increase in body mass during the course of extended treatment, an expected consequence of increased age, but the relative EDL, tibialis anterior and soleus muscle masses were similar across all groups (0.037±0.002, 0.159±0.002 and 0.027±0.001% body mass, respectively; n.s. among groups and for time).

Do vasodilators have similar effects, irrespective of their mode of action?

Compared to controls (0.40±0.01 $ml.min^{-1}$), a significantly greater femoral blood flow (FBF) was seen for prazosin (0.59±0.0.02 $ml.min^{-1}$), verapamil (0.59±0.02 $ml.min^{-1}$), cromakalim (0.57±0.01 $ml.min^{-1}$) and ethanol (0.59±0.03 $ml.min^{-1}$) treated mice after 14 d (all $P<0.01$; Fig. 1A). A significantly higher C:F was seen in EDL of prazosin (1.62±0.05), verapamil (1.60±0.01), cromakalim (1.57±0.06), and ethanol (1.57±0.07)

treated mice (all $P<0.001$ *vs.* control of 1.20±0.01, Fig. 1B). As blood flow velocity and microvascular diameter were not measured, 'shear stress-induced angiogenesis' is an assumption based on extrapolation from previous studies from our group that gathered such data (e.g. (Hudlická and Brown, 2009)). Electron microscope images taken after 14 d of treatment revealed that capillaries from the four vasodilatory groups displayed a control phenotype, indicating that little or no angiogenesis was occurring at this time point; i.e. no luminal processes, no vacuolisation, and no thinning of the basement membrane (*cf* (Williams et al., 2006a); data not shown), suggesting that active angiogenesis was complete by 14 days. The increase in C:F was proportional to the increase in FBF among the different treatment groups (Fig. 2). We tested the effects of age on a few animals from later groups that suggested the small difference in C:F between control and 42DR was likely age-related (data not shown).

[Fig. 1 near here]

[Fig. 2 near here]

A variable effect of nitric oxide and prostaglandin inhibition

Following L-NNA blockade of NOS activity, femoral blood flow for all vasodilator groups were either at (cromakalim, ethanol) or below (prazosin, verapamil) control levels (all $P<0.001$ *vs.* vasodilator alone; data not shown). With co-administration of indomethacin flow was reduced by 53% *vs.* prazosin alone, 56% *vs.* verapamil, 36% *vs.* cromakalim, and 46% *vs.* ethanol (all $P<0.05$; data not shown).

With L-NNA, C:F remained at control levels in prazosin (1.213±0.023) and verapamil (1.269±0.031) groups (both $P<0.001$ *vs.* vasodilator; n.s. *vs.* control), but with cromakalim (1.353±0.054) and ethanol (1.369±0.036) there was only a ~60%

lower C:F than with vasodilator alone (both $P<0.05$ *vs.* dilator and control). Co-administration of indomethacin with the various vasodilators led to a lower C:F in all groups compared with vasodilator alone: 73% reduction *vs.* prazosin (1.313±0.043, $P<0.001$), 36% reduction *vs.* verapamil (1.482±0.062, $P<0.001$), 63% reduction *vs.* cromakalim (1.340±0.024, $P<0.05$), and 23% reduction *vs.* ethanol (1.524±0.039, n.s.) (Fig. 3).

In the cromakalim and ethanol groups, L-NNA and indomethacin were also given in combination to assess the effects of dual inhibition of NO and prostaglandin synthesis. Then, C:F was not significantly different to control levels (1.210±0.007 and 1.247±0.029, respectively).

We conducted a few experiments (two animals for each) to check there were no obvious effects of the inhibitors alone on femoral blood flow, blood pressure and C:F (consistent with previous finding using rats). As with age-matched controls these data are omitted as the statistical treatment was not robust due to small sample size.

[Fig. 3 near here]

Time course of hindlimb blood flow responses, shear-induced angiogenesis and capillary regression

Femoral blood flow (FBF) increased with length of prazosin treatment, and a positive correlation between FBF and C:F was seen up to 4 wk prazosin treatment (Fig. 4). After cessation of treatment, FBF was reduced from a peak of 0.59ml.min^{-1} to 0.54ml.min^{-1} ($P<0.05$) within 7 d, and returned to control values 2-6 wk later.

C:F of EDL increased within 1 wk of prazosin treatment with C:F reaching a presumed peak of 1.62±0.03 by 2 wk; this was maintained for the 4 weeks of

treatment. Within 1 wk after prazosin withdrawal, C:F approached control values in EDL (Fig. 4).

[Fig. 4 near here]

Reciprocal biochemical responses to angioadaptation

The prazosin-induced rise in FBF was accompanied by a steady decline in EDL muscle VEGF protein from control levels (0.21±0.02) that continued after treatment was stopped and blood flow declined, reaching its lowest level at 14 d recovery (14DR) (0.14±0.02; P=0.058), but recovered to control levels after 6 wk (0.19±0.01, n.s.). During the same period, basal levels of VEGFR2 (Flk-1) protein (0.14±0.02) rose to a peak at 4 wk prazosin treatment (0.25±0.06; P=0.117), and were maintained for some time after cessation of vasodilator treatment (6 wk post-prazosin = 0.24±0.03; n.s. *vs.* 4 wk prazosin). VEGFR1 (Flt-1) levels mirrored those of Flk-1 (data not shown). Levels of eNOS protein increased with treatment, and continued to rise until 7DR (0.12±0.02 *vs.* control of 0.08±0.02; P<0.05). A subsequent decrease in eNOS expression followed the decline in FBF, returning to control values at 42DR (0.07±0.02; n.s.). Levels of eNOS varied inversely with respect to VEGF (Fig. 5).

[Fig. 5 near here]

Ang-1 protein levels rose sharply from control values (0.13±0.02) to maximum at 3DR (0.31±0.06; P=0.09; Fig. 6). A more gradual decline was then seen, but even by 42DR expression had not reached control values (0.23±0.03). Conversely, TSP-1 levels decreased relative to control control from 14DP, and this decrease was

maintained until 42DR. Levels of the Ang-1 receptor, Tie-2, did not change (n.s. *vs.* control).

[Fig. 6 near here]

Discussion

Vasodilators have similar angiogenic effects irrespective of their mode of action

We demonstrate an increase in C:F of mouse EDL by ~30% with all vasodilators tested, accompanying a consistent increase in femoral blood flow (FBF) of ~45%. Reversal of these responses on withdrawal of vasodilator treatment demonstrates a positive correlation between hindlimb blood flow and muscle capillary supply. These data support the hypothesis that angiogenesis occurs primarily due to elevated microvascular shear stress, not an independent action of vasodilator compounds (Egginton et al., 2001). Interestingly, as the increase seen within the four vasodilator groups occurred to a similar level (C:F ~1.6), and capillary phenotype appeared similar to control at 14 d (unpublished data), the extent of angiogenesis could be the maximum inducible by elevated shear stress. A lack of statistical difference for C:F between 14DP and 28DP suggests there is an upper limit to skeletal muscle capillary supply (Snyder, 1987). The appearance of capillaries undergoing longitudinal splitting in mouse EDL following prazosin-induced hyperaemia have previously been described, and the extent of angiogenesis varies with FBF, with a maximal response induced by 28 d (Williams et al., 2006a). This muscle was chosen because of its mixed fibre type composition, so qualitatively, but not quantitatively similar effects may be expected in other muscles. The rapidity of response in both growth and rarefaction is consistent with capillary splitting being energetically efficient compared

with the more common sprouting angiogenesis, as evidenced by the low proliferation labelling index of endothelial cells (Egginton, 2011).

The role of nitric oxide in angiogenesis

Nitric oxide (NO), generated by nNOS in muscle fibres (Balon and Nadler, 1994) or eNOS in blood vessel endothelium in response to increased shear stress (Fleming and Busse, 2003), contributes to the hyperaemia observed during acute muscle contractions in animals (Hirai et al., 1994) and humans (Boushel, 2003, Schrage et al., 2004). Both isoforms can be upregulated when muscle activity is increased long-term by electrical stimulation (nNOS; (Reiser et al., 1997)) or exercise training (eNOS; (Sun et al., 1994)). NO contributes to the basal tone and diameter of arterioles in rat skeletal muscle (Kaley et al., 1992, Friebel et al., 1995) and to the dilatation of small arterioles during acute contractions (Cohen et al., 2000).

Complete inhibition of angiogenesis by L-NNA was seen in two of the four vasodilator groups (prazosin, verapamil), where hindlimb blood flow was slightly below control levels, strongly suggesting that the role of NO in shear-induced angiogenesis is as a vasodilator. Similarly, L-NNA inhibition of NOS in rats abolished the increase in measured shear stress (Hudlická et al., 2006), along with cell proliferation and capillary growth in electrically stimulated muscles (Milkiewicz et al., 2001). We therefore speculate that the angiogenesis caused by prazosin and verapamil was a consequence of NO-dependent increase in flow. In the remaining two groups (cromakalim, ethanol) L-NNA only partially inhibited the increase in C:F, and with FBF at control levels following NOS inhibition demonstrates the contribution of NO-independent stimulators of angiogenesis.

Prostaglandins and angiogenesis

Prostaglandin production is involved in endothelial cell migration (Tsujii et al., 1998) and tubular network formation in 3D collagen gels (Milkiewicz et al., 2006). Prostacyclin (PGI_2) (Frangos et al., 1985) and cyclooxygenase-II (COX-2) (Topper et al., 1996) are released in response to an increase in shear stress, and modulate the expression of angiogenic genes within human endometrium (Gately and Li, 2004) such as VEGF, bFGF, Ang-1 and Ang-2 (Kamio et al., 2008, Smith et al., 2006). COX-2 inhibition during vasodilator treatment gave a variable effect on muscle capillarity (73-91%) but a similarly reduced femoral blood flow (75-81%). Unlike NOS inhibition, however, the reduction in FBF following prostaglandin synthesis blockade cannot account for the large inhibitory action on angiogenesis. This indicates that a prostaglandin may have direct angiogenic actions in the capillary splitting phenotype, but that this varies depending on the vasodilatory mechanism involved (prazosin and cromakalim > verapamil and ethanol). However, the absence of a shear stimulus following NOS blockade suggests inhibition of not only the NO contribution of the flow stimulus, but also the prostaglandin-mediated portion. Indeed, as there is considerable cross-talk between the two systems it is likely that inhibition of one vasodilator affects the other (Rubanyi et al., 1986)

Co-administration of both L-NNA and indomethacin was needed to effect a complete blockade of angiogenesis with ethanol and cromakalim, supporting the combined effect of NO and prostaglandin activity. Acute intravascular administration of cromakalim into rats led to a significant dose–dependent decrease in both mean arterial pressure and hindlimb perfusion pressure, an effect that was greatly reduced on blockade of NO production (Jahr et al., 1994). Interestingly, flow during ethanol and cromakalim treatments with NO blockade were at control levels, yet angiogenesis was observed. Ethanol has been shown to increase mRNA expression of a number of

growth factors and receptors in rat skeletal muscle at rest and after acute exercise, including VEGF, TGF-β1, bFGF and VEGFR1 (Gavin and Wagner, 2002). Induction of one or more of these may provide a compensatory angiogenic response, irrespective of a flow stimulus, although the form of angiogenesis induced is unknown. Clearly, changes in capillary supply with vasodilator treatment will alter the surface area for expression of EC markers, requiring some caution in interpretation of 'whole muscle' protein levels. However, laser micro-dissection of capillaries has been shown to generate parallel data to that derived from whole muscles (Milkiewicz and Haas, 2005), suggesting we may have confidence in the conclusions drawn.

The time course of angioadaptation

Having established a similar response to various vasodilators, suggesting that angiogenesis is due to mechanical forces associated with increased blood flow, though not necessarily in signalling mechanisms, we then used the most commonly used vasodilator to test whether such a response involves a turnover rate appropriate for previously detailed low mitotic activity. Prazosin may be a relatively selective vasodilator (Egginton, 2009) with little or no secondary angiogenic effects on the systemic vasculature (Hudlická, 1998), and results in angiogenesis within EDL that was complete by 4 wk of treatment (Williams et al., 2006a). The present study demonstrates that hindlimb blood flow increased with treatment duration, presumably resulting in a progressively greater angiogenic shear stress stimulus in the downstream microvasculature, likely inducing capillary splitting. In addition, arteriogenesis was evident by 4 wk prazosin treatment (data not shown) and it is likely that arteriolisation of capillaries, and terminal arteriole growth, allows better haemodynamic regulation of flow commensurate with increased capillarisation

(Hansen-Smith et al., 1998). A positive feedback loop involving decreased tissue resistance and increased blood flow may then follow. Femoral blood flow decreased by 25% within 1 wk of cessation of prazosin treatment, after which flow returned to normal values, consistent with regression of newly formed microvessels. As femoral blood flow reflects perfusion of all hindlimb muscles, the angiogenic profile of a large extensor muscle (EDL) may be indicative of a general response. Angiogenesis was complete by 2 wk prazosin treatment (above), and C:F returned toward control levels within 1-2 wk, indicating rapid capillary regression. The rapidity of growth without significant EC turnover indicates a non-proliferative expansion of the capillary bed, likely by elongation of existing microvessels. Given the presumed energy-efficient reduced structural remodelling during expansion, it follows that regression may be less demanding than following sprouting and hence a similarly quick response is consistent with this form of capillary growth.

Biochemical responses induced by elevated shear stress

In addition to the complexities of VEGF involvement (Wagner, 2011) the pro- and anti-angiogenic responses to short–term exercise and withdrawal have only recently been reported (Olenich et al., 2014, Hellsten and Hoier, 2014), but parallel studies on blood flow have not been conducted.

1. VEGF expression is inversely related to blood flow

Many studies report an upregulation of VEGF following both acute and chronic increases in shear stress (Da Silva-Azevedo et al., 2002) or muscle activity (Milkiewicz et al., 2005, Gavin and Wagner, 2002). Sequestration of this key angiogenic cytokine blocks angiogenesis in response to muscle overload and hyperaemia, both of which lead to a rise in VEGF levels *in vivo* (Williams et al.,

2006b). A rise in VEGF expression is reported within the first few days of prazosin treatment, followed by a reduction in protein levels after 4 wk (Egginton et al., 2011, Rivilis et al., 2002). A decline in VEGF levels on cessation of treatment would mediate microvascular regression in line with its anti-apoptotic role (Ferrara, 2001). The present study used samples taken after the initial transient rise, so levels of VEGF declined in response to chronic prazosin treatment that continued after withdrawal, subsequently increasing after 14DR and reaching control levels only after 6 wk. This may be influenced by differences in blood oxygen supply, as VEGF synthesis is oxygen-dependent (Ferrara et al., 2003). Thus, a decrease in VEGF protein in the presence of prazosin may be a consequence of 'luxury perfusion' (Baum et al., 2004), with subsequent microvascular regression on cessation of prazosin treatment providing a resurgent drive for VEGF synthesis. While it is likely that hyperoxia, should it occur during hyperaemia, would not elicit the opposite response to hypoxia, delivery of oxygen at the microvascular level will be elevated by increased capillary perfusion, thereby increasing the PO_2 gradient. However, this possibility requires further research as 'sedentary' mice selectively bred to have high aerobic exercise capacity with concomitant elevation in C:F compared to normal mice do not exhibit lower VEGF, but rather unchanged (or higher) basal VEGF protein (Audet et al., 2011).

2. Flk-1 expression tracks changes in blood flow

Extracellular growth factor receptors such as Flk-1, the cognate receptor for VEGF predominantly expressed on endothelium and most associated with angiogenesis (Neufeld et al., 1999), are implicated in the shear stress response (Wang et al., 2002) where endothelial mechanotransduction modulates expression of shear stress responsive genes (Resnick et al., 1997). VEGF expression can increase in hypoxic

(Wang et al., 2007) and ischaemic (Milkiewicz et al., 2003) conditions, although in this study it is most likely to result from increases in shear stress. Flk-1 upregulation is important *in vivo* for the endothelium to withstand increases in shear stress (Conway and Schwartz, 2012) and higher levels after prazosin treatment may compensate for reduced ligand availability, with subsequent decreases likely tracking reduction in shear stress and augmented by capillary regression. Hence, a Flk-1 mediated, ligand-independent mechanotransduction may contribute more significantly to this form of angiogenesis than VEGF *per se* (Wang et al., 2002, Baum et al., 2004), offering a parallel with the situation in low-flow ischaemia (Milkiewicz et al., 2004).

3. Levels of eNOS are inversely related to levels of shear stress

Increases in shear stress upregulates expression of the endothelial-specific isoform of NOS enzyme, hence prazosin treatment leads to activation of the eNOS gene (Williams et al., 2006c), and perhaps eNOS phosphorylation by a glycocalyx-linked deformation of caveolae (Pahakis et al., 2007). Importantly, the present study demonstrates that a decreased shear stress after cessation of vasodilator therapy leads to a decrease in eNOS expression, a key mediator of shear stress-induced angiogenesis: vessel growth is absent in eNOS knockout mice (Williams et al., 2006a). It may appear paradoxical that levels of VEGF decreases during significant blood vessel growth but eNOS upregulation increases NO production, which itself is angiogenic (Baum et al., 2004) so the downstream effects of NO may compensate for a decline in VEGF levels, e.g. angiogenesis appears to be NO-dependent in ischaemic tissue (Murohara et al., 1998). Indeed, local paracrine interactions were evident in mice, where flow-mediated endothelial stimulation of myocyte VEGF production was paralleled by myocyte-derived VEGF supporting microvascular remodelling (Uchida et al., 2015).

4. Complementary changes in angiopoietins

Ang-1 promotes vessel integrity through pericyte recruitment and endothelial quiescence by apoptosis suppression (Babaei et al., 2003, Carmeliet, 2003). Levels more than doubled after just 2 wk of prazosin treatment, but did not decline after the end of treatment. Pro-angiogenic effects of Ang-1 have also been demonstrated in cultured coronary artery ECs (Chen et al., 2004) and subcutaneous Matrigel plugs (Babaei et al., 2003), dependent on the action of NO. Hence, an upregulation of Ang-1 in parallel with eNOS may stimulate blood vessel growth, while any decline may contribute to prevention of EC apoptosis, but *in vivo* changes are modest compared with those expected from *in vitro* studies. Ang-2 displays pleiotropic, VEGF-dependent effects that promote microvascular growth, as Ang-2/Tie2 interactions destabilise the endothelium (Hanahan, 1997). Again, *in vitro* studies suggest that elevated shear stress downregulates Ang-2 expression (Goettsch et al., 2008), but the levels of shear used were not physiological and may reflect EC dysfunction. However, we saw no significant change in Ang-2 levels during angiogenesis or microvessel regression (data not shown), also little or no change in expression of its cognate receptor, Tie-2. These findings suggest that, in physiological shear-induced angiogenesis, Ang-2 elevation may be unnecessary in the presence of pro-angiogenic Ang1 levels. In the absence of angiopoietin stimulation, therefore, the levels of Tie-2 may be 'diluted' on EC expansion relative to other EC markers, such as Flk-1.

5. Prolonged depression of an anti-angiogenic factor

TSP-1 is downregulated in response to increases in shear (Bongrazio et al., 2006), and, when complexed with its receptor, CD36, inhibits VEGFR2 (Chu et al., 2013). This reduces angiogenesis readouts *in vitro* (Klenotic et al., 2013) and *in vivo* (Audet

et al., 2013). As in this study, a downregulation of pro-angiogenic factors has previously been seen during muscle denervation and capillary regression (Wagatsuma et al., 2005), but anti-angiogenic factors such as the thrombospondins may also play a role in maintaining an effective control of capillary growth (Olfert et al., 2006). Changes in capillarisation are then dependent on the balance between pro- and anti-angiogenic factors (Carmeliet, 2003), consistent with the lack of a rebound rise in TSP-1 after cessation of vasodilator treatment. However, capillary rarefaction from basal levels may represent a different (pathological) context than capillary regression to basal levels after (physiological) capillary growth, likely representing active signalling and withdrawal of signal, respectively.

Conclusions and integrative perspective

Vasodilator-induced angiogenesis occurs in response to mechanical forces associated with increased blood flow, i.e. is not a chemotransductive response. An elevated microvascular shear stress using common vasodilators increases muscle C:F to a similar extent, allowing us to examine the mechanotransductive response, including evidence for altered expression of pro- and anti-angiogenic proteins. Further alterations in protein expression associated with capillary regression occur when the vasodilator is withdrawn. Interestingly, NO or COX inhibitors prevented the effects of vasodilators on C:F, although these pathways are not solely responsible for vasodilatation. An increase in C:F paralleled that of FBF, suggesting both arteriogenic and angiogenic components of an integrated response to elevated shear stress, while subsequent reduction leads to vessel regression, supporting the contention that angiogenesis is induced by mechanotransduction following elevated shear stress. A conceptual summary of the main findings is provided (Suppl. Fig. 1).

Post-transcriptional modification of proteins may be required before they become biologically active, cytokines may be stored intracellularly as inactive precursors until phosphorylated (Goettsch et al., 2008) (Ferrara, 2001), and VEGF supplementation is ineffective in the absence of eNOS (Murohara et al., 1998). Hence, further studies are required to document mechanistic regulation *in vivo*. A brief temporal delay between modulation of mRNA expression and an identifiable change in protein levels is to be expected. However, The biological effects continued for some time after treatment ended, e.g. FBF and VEGF levels, likely due to prazosin clearance time and kinetics of tissue remodelling. Prolonged increases in blood flow and shearing forces may perpetuate the proteomic response, while structural changes accompanying microvascular expansion or regression likely outlast the biochemical effects of chronic vasodilatation.

Clinical relevance and application

An increase in shear stress is one of the many factors thought to induce blood vessel growth in response to exercise (Prior et al., 2004), including signals effective over different time courses such as altered enzymatic activity (Mujika and Padilla, 2001) and mechanical stretch (Prior et al., 2004, Williams et al., 2006a). While VEGF expression decreases following prazosin-induced chronic hyperaemia, it rises during acute increases in blood flow associated with periods of activity (Prior et al., 2004). During periods of disuse regression of capillaries is associated with muscle atrophy (Dedkov et al., 2002), despite reduced vasomotor tone (Tyml et al., 1999). Increased blood flow reduces the extent of capillary regression, such that maintenance of blood flow during periods of inactivity may attenuate the extent of microvascular rarefaction, hence exercise regimes are important during periods of bed rest and spaceflight (Trappe et al., 2001). Supplementation of different growth factors may

provide acute benefit during recovery from surgery, especially if exercise is not possible, but risk side effects. Alternative therapies could involve controlled use of vasodilators, as rapidity of response predicted from an energetically efficient (low EC mitosis) form of angiogenesis offers temporal selectivity. NO supplementation, manipulation of the eNOS/NO system or Ang-1 and Flk-1 may also be effective. VEGF treatment may be contraindicated in this scenario, as its effect on vascular permeability produces oedema (Ferrara, 2001), but suppression of thromobospondin 1 may be beneficial.

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Conflict of interest

The authors confirm there are no conflicts of interest.

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Supplementary Table 1.

Group	Femoral flow (ml/min)	Blood pressure (mmHg)	Heart rate (min^{-1})
Control	0.40±0.012	91.3±3.26	311±38.9
Prazosin 2d	0.51±0.043	99.0±1.16	257±19.5
Prazosin 4d	0.48±0.019	82.3±2.56	306±13.8
Prazosin 7d	0.54±0.075	93.5±3.40	310±25.5
Prazosin 14d	0.57±0.029	88.5±3.29	360±16.6
Prazosin 28d	0.61±0.024	90.2±4.18	342±22.8
1wk regression	0.54±0.010	87.1±4.14	296±18.0
2wk regression	0.44±0.026	86.0±3.85	289±15.5
6wk regression	0.40±0.020	84.3±2.33	248±23.3

NB. Given that mice consume ~3 ml of water each day, this equates to ~150 µg of prazosin daily, a dose known to stimulate shear stress-induced angiogenesis in both rats and mice (Hudlická, 1998) (Baum et al., 2004).

Legends to figures

Figure 1. (A) Femoral blood flow after 14 d chronic vasodilator treatment. (B) C:F in EDL of mice after 14 d chronic vasodilator treatment. The latter estimate used a 270x270µm counting square and an unbiased sampling protocol, aiming for at least 50 fibres or 100 capillaries to minimise statistical errors (Egginton, 1990a, Egginton, 1990b). Mean±SEM (n=6). ** $P<0.01$ *vs*. control. Abbreviations: FBF, femoral blood flow; C:F, capillary to fibre ratio; Cont, control; Praz, prazosin; Ver, verapamil; Crom, cromakalim; Eth, ethanol.

Figure 2. Individual femoral flow *vs.* C:F values showing a positive correlation among animals; controls are circled (R^2= 0.8412; Y=1.752*X+0.5330)

Figure 3. Left panel: C:F after 14 d chronic vasodilator treatment with blockade of NO and prostaglandin production. Mean±SEM (n=6). *$P<0.05$ and **$P<0.001$ *vs.* control, $^{+}P<0.05$ and $^{++}P<0.001$ *vs.* vasodilator alone. Abbreviations: P, prazosin; V, verapamil; C, cromakalim; E, ethanol; LNNA, N_w-nitro-L-arginine; Indo, indomethacin. Right panel: Example micrographs of lectin stained cryosections for i) control, ii) control + LNNA, iii) prazosin, iv) prazosin + LNNA, v) verapamil, vi) ethanol, vii) cromakalim, viii) cromakalim + LNNA. Scale bar = 150µm.

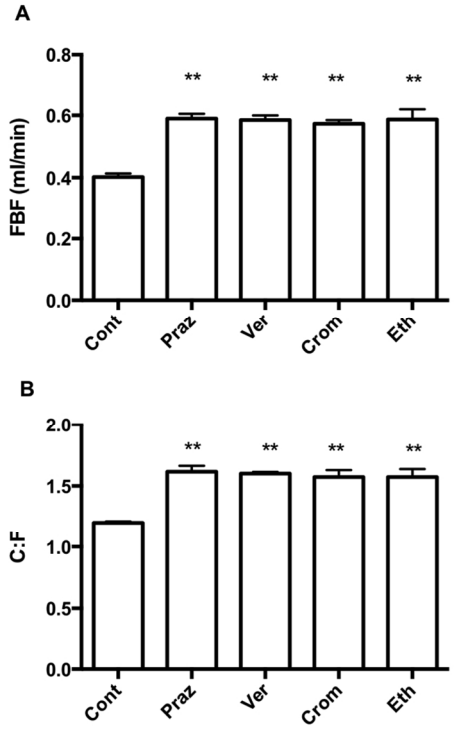
Figure 4. Femoral blood flow (solid line) and C:F before and during chronic prazosin treatment (open bars), and 1-6 wk after cessation of treatment (hatched bars). Mean±SEM (n=6). The evident relationship between blood flow and capillarisation is quantified as follows: angiogenesis Cont – 28DP r=0.877; regression 7DR-42DR r=0.896. FBF: *$P<0.05$ *vs*. control, **$P<0.01$ *vs*. control, ***$P<0.001$ *vs*. control,

$^{+}P<0.05$ *vs*. prazosin 14 d and 28 d. C:F $^{\#}P<0.05$ *vs*. control, $^{\$}P<0.05$ *vs*. prazosin 14 d and 28 d. Abbreviations: DP, days of prazosin treatment; DR, days since prazosin removal.

Figure 5. Relative protein expression for VEGF (triangle), Flk-1 (square) and eNOS (filled circle) in mouse EDL over 4 wk prazosin treatment, and 6 wk recovery, correlated with femoral blood flow (open circle). Densitometry values were referenced to total protein content, and individual protein levels expressed in arbitrary units (AU). *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs.* control (FBF). $, + and # $P<0.05$ *vs.* control (Flk-1, VEGF and eNOS, respectively). *NB* some error bars are contained within the symbols. Abbreviations: WP, weeks of prazosin treatment.

Figure 6. Changes in Tie-2, Ang-1 and TSP-1 protein levels in mouse EDL during angioadaptation. Note different scale to Fig. 5. *$P<0.05$ *vs.* control (TSP-1).

Supplementary Figure 1. Conceptual summary of the study design and main findings.



(A) Femoral blood flow after 14 d chronic vasodilator treatment. (B) C:F in EDL of mice after 14 d chronic vasodilator treatment. The latter estimate used a 270x270µm counting square and an unbiased sampling protocol, aiming for at least 50 fibres or 100 capillaries to minimise statistical errors (Egginton, 1990a, Egginton, 1990b). Mean±SEM (n=6). ** P<0.01 vs. control. Abbreviations: FBF, femoral blood flow; C:F, capillary to fibre ratio; Cont, control; Praz, prazosin; Ver, verapamil; Crom, cromakalim; Eth, ethanol.
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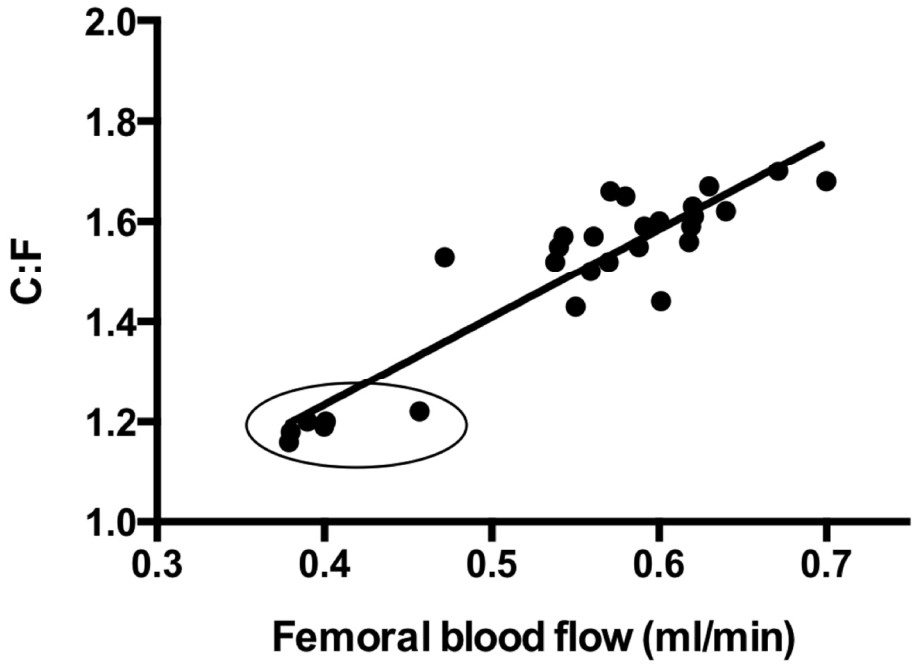
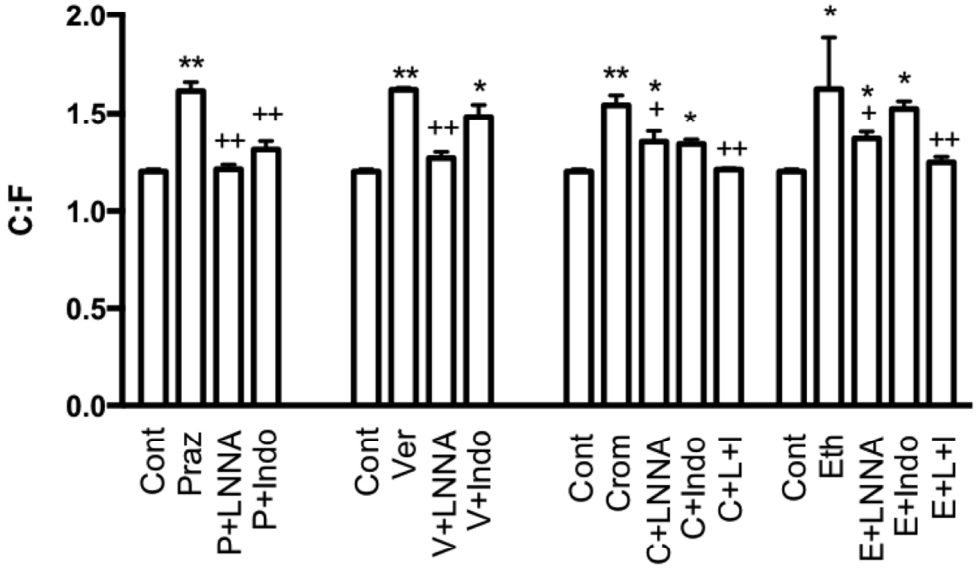


Individual femoral flow vs. C:F values showing a positive correlation among animals; controls are circled
(R2= 0.8412; Y=1.752*X+0.5330)
508x381mm (72 x 72 DPI)



Left panel: C:F after 14 d chronic vasodilator treatment with blockade of NO and prostaglandin production. Mean±SEM (n=6). *P<0.05 and **P<0.001 vs. control, +P<0.05 and ++P<0.001 vs. vasodilator alone. Abbreviations: P, prazosin; V, verapamil; C, cromakalim; E, ethanol; LNNA, Nw-nitro-L-arginine; Indo, indomethacin.
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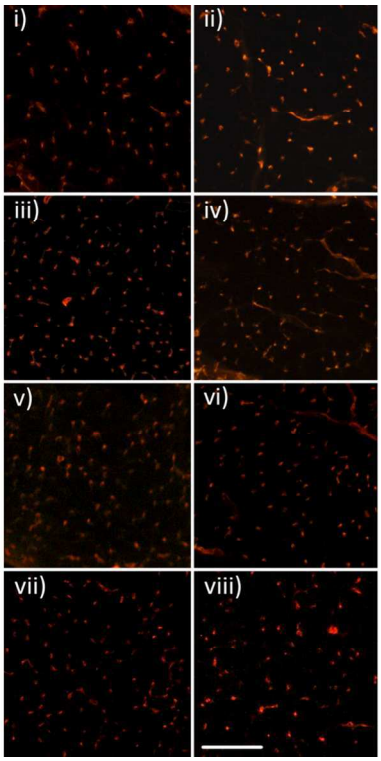

Right panel: Example micrographs of lectin stained cryosections for i) control, ii) control + LNNA, iii) prazosin, iv) prazosin + LNNA, v) verapamil, vi) ethanol, vii) cromakalim, viii) cromakalim + LNNA. Scale bar = 150μm.
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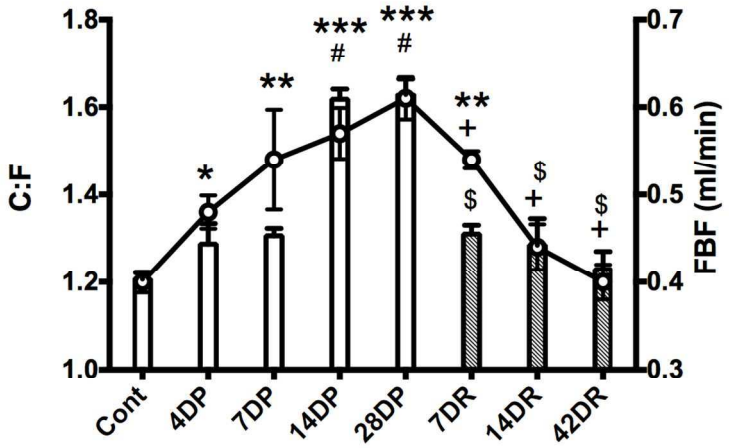

Femoral blood flow (solid line) and C:F before and during chronic prazosin treatment (open bars), and 1-6 wk after cessation of treatment (hatched bars). Mean±SEM (n=6). The evident relationship between blood flow and capillarisation is quantified as follows: angiogenesis Cont – 28DP r=0.877; regression 7DR-42DR r=0.896. FBF: *P<0.05 vs. control, **P<0.01 vs. control, ***P<0.001 vs. control, +P<0.05 vs. prazosin 14 d and 28 d. C:F #P<0.05 vs. control, $P<0.05 vs. prazosin 14 d and 28 d. Abbreviations: DP, days of prazosin treatment; DR, days since prazosin removal.
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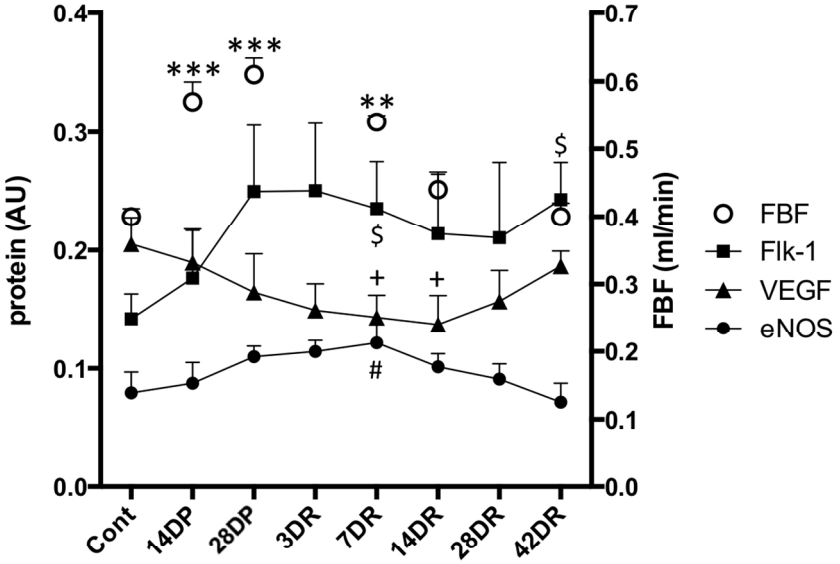

Relative protein expression for VEGF (triangle), Flk-1 (square) and eNOS (filled circle) in mouse EDL over 4 wk prazosin treatment, and 6 wk recovery, correlated with femoral blood flow (open circle). Densitometry values were referenced to total protein content, and individual protein levels expressed in arbitrary units (AU). *P<0.05, **P<0.01, ***P<0.001 vs. control (FBF). $, + and # P<0.05 vs. control (Flk-1, VEGF and eNOS, respectively). NB some error bars are contained within the symbols. Abbreviations: WP, weeks of prazosin treatment.
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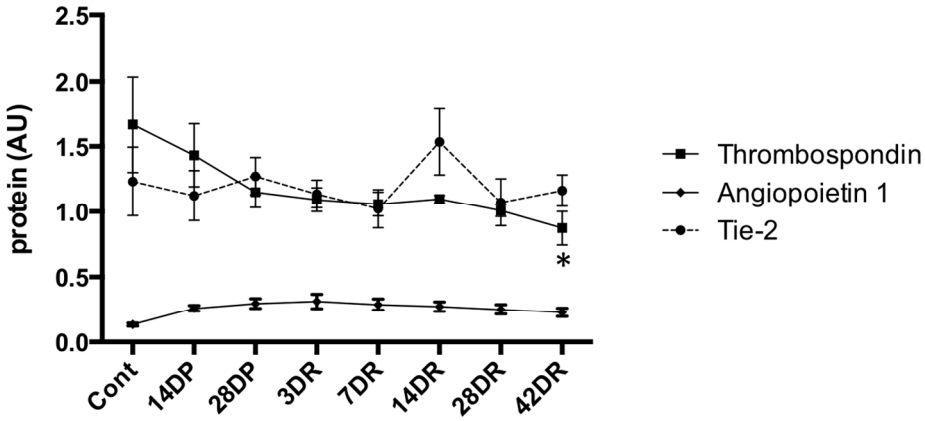

Changes in Tie-2, Ang-1 and TSP-1 protein levels in mouse EDL during angioadaptation. Note different scale to Fig. 5. *P<0.05 vs. control (TSP-1).
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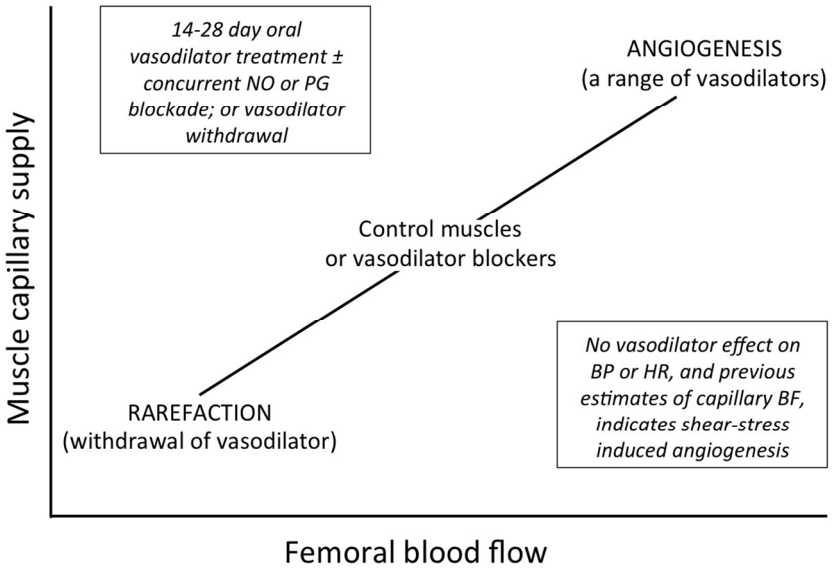

Conceptual summary of the study design and main findings.
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