The Journal of Physiology

Daily muscle stretching enhances blood flow, endothelial function, capillarity, vascular volume and connectivity in aged skeletal muscle

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Edited by: Michael Hogan & Ylva Hellsten

Key points

- In aged rats, daily muscle stretching increases blood flow to skeletal muscle during exercise.
- Daily muscle stretching enhanced endothelium-dependent vasodilatation of skeletal muscle resistance arterioles of aged rats.
- Angiogenic markers and capillarity increased in response to daily stretching in muscles of aged rats.
- Muscle stretching performed with a splint could provide a feasible means of improving muscle blood flow and function in elderly patients who cannot perform regular aerobic exercise.

Abstract Mechanical stretch stimuli alter the morphology and function of cultured endothelial cells; however, little is known about the effects of daily muscle stretching on adaptations of endothelial function and muscle blood flow. The present study aimed to determine the effects of daily muscle stretching on endothelium-dependent vasodilatation and muscle blood flow in aged rats. The lower hindlimb muscles of aged Fischer rats were passively stretched by placing an ankle dorsiflexion splint for 30 min day^{-1}, 5 days week^{-1}, for 4 weeks. Blood flow to the stretched limb and the non-stretched contralateral limb was determined at rest and during treadmill exercise. Endothelium-dependent/independent vasodilatation was evaluated in soleus muscle arterioles. Levels of hypoxia-induced factor-1α, vascular endothelial growth factor A and neuronal nitric oxide synthase were determined in soleus muscle fibres. Levels of endothelial nitric oxide synthase and superoxide dismutase were determined in soleus muscle arterioles, and microvascular volume

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DOI: 10.1113/JP275459

and capillarity were evaluated by microcomputed tomography and lectin staining, respectively. During exercise, blood flow to plantar flexor muscles was significantly higher in the stretched limb. Endothelium-dependent vasodilatation was enhanced in arterioles from the soleus muscle from the stretched limb. Microvascular volume, number of capillaries per muscle fibre, and levels of hypoxia-induced factor-1α, vascular endothelial growth factor and endothelial nitric oxide synthase were significantly higher in the stretched limb. These results indicate that daily passive stretching of muscle enhances endothelium-dependent vasodilatation and induces angiogenesis. These microvascular adaptations may contribute to increased muscle blood flow during exercise in muscles that have undergone daily passive stretch.

(Received 20 October 2017; accepted after revision 19 February 2018)
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Introduction

Ageing alters muscle blood flow during exercise, independent of changes in cardiac output, resulting in a mismatch between oxygen supply and demand (Rodeheffer *et al.* 1984; Delp *et al.* 1998; Behnke *et al.* 2012). Ageing-induced alterations of muscle blood may be linked to the impairment of endothelium-dependent vasodilatation of resistance arteries (Muller-Delp *et al.* 2002) or changes in muscle capillarity, which are dependent on fibre size and oxidative capacity (Hepple & Vogell, 2004; Groen *et al.* 2014; Barnouin *et al.* 2017) in skeletal muscle. Exercise training, even initiated in late life, enhances endothelium-dependent dilatation (Spier *et al.* 2004, 2007), increases capillarity (Charifi *et al.* 2004; Jensen *et al.* 2004), and improves blood flow distribution and capacity in the aged lower limb (Behnke *et al.* 2012). Despite the well-known beneficial effects of exercise, the rate of compliance to exercise training programmes is low in elderly people (Chrisman *et al.* 2015), often as a result of the strenuous nature of exercise training. Reduced mobility and diminished muscular strength may decrease adherence to exercise programmes in the elderly (Morie *et al.* 2010); therefore, it is important to develop alternative therapies that mitigate the loss of muscle blood flow in the elderly.

Muscle stretching is widely performed as a warm-up or cool-down for patients undergoing physical therapy (Katalinic *et al.* 2011; Apostolopoulos *et al.* 2015). The intensity of muscle stretching is relatively light compared to aerobic exercise, such that even very old individuals can perform muscle stretching with minimal risk of injury. It has been reported that muscle stretching prevents muscle atrophy in immobilized lower limbs (Agata *et al.* 2009); however, little is known about the effects of daily muscle stretching on limb blood flow. Mechanical stimuli have a crucial role in keeping endothelial cells healthy (Fujiwara, 2003); endothelial cells sense stretch and shear stress, resulting in alterations of gene expression, morphology and function (Naruse *et al.* 1998; Kuebler *et al.* 2003; Thacher *et al.* 2010). Responses to the mechanical stimuli of stretch and shear stress also play important roles in maintaining normal vascular function and their impairment leads to various vascular diseases (Thijssen *et al.* 2009; Ando & Yamamoto, 2011). Circumferential stretch has been demonstrated to increase endothelial nitric oxide synthase (Awolesi *et al.* 1995) and to release nitric oxide (NO) from endothelial cells (Kuebler *et al.* 2003). Mechanical stretch/overload has been shown to increase levels of vascular endothelium-derived growth factor (VEGF) and increase the capillarization of rat skeletal muscle (Rivilis *et al.* 2002). In healthy young humans, passive leg movement increases muscle blood flow and augments interstitial VEGF and endothelial nitric oxide synthase (eNOS) mRNA in muscle, independent of changes in metabolism or central haemodynamics (Hellsten *et al.* 2008; McDaniel *et al.* 2012); however, the effects of daily passive muscle stretching on adaptations of microvascular function and angiogenesis have not been studied.

We hypothesized that daily passive stretching of muscle using a splint (Baewer *et al.* 2004) would improve endothelium-dependent vasodilatation and increase exercise-induced hyperaemia in the skeletal muscle of aged rats. To determine whether acute changes in blood flow during daily muscle stretching provide the signal for microvascular adaptations as the muscle undergoes daily stretching, we evaluated blood flow responses during and immediately after an acute bout of static muscle stretching. After 4 weeks of daily muscle stretching, we evaluated muscle blood flow at rest and during exercise, and assessed endothelium-dependent dilatation, capillarity and markers of angiogenesis in skeletal muscle.

Methods

Ethical approval

Twenty-month-old male Fisher 344 rats (n = 68) were obtained from National Institute of Aging. For

performance of daily stretching, rats were assigned to stretch ($n = 35$), cage control ($n = 8$) or sham control ($n = 8$) groups. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Florida and Florida State University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All rats were housed in a temperature/light-controlled environment and given access to standard rat chow and water *ad libitum*.

Muscle stretching protocol

The splints were made from Polyform (Smith & Nephew Rolyan, Inc., Menomonee Falls, WI, USA), as described previously (Baewer *et al.* 2004). The splint material was rendered malleable by heating in 70°C water and shaped by hand to fit onto the back of the leg and bottom of the foot. Muscle stretching was performed for 30 min, 5 days week^{-1}, for 4 weeks by applying the splint to hold the left ankle joint at 30° of dorsiflexion (Fig. 1) as developed by Baewer *et al.* (2004). Placement of this splint produced stretch in muscles of the back of the lower leg: the soleus, plantaris, flexor hallucis longus and flexor digitorum longus muscles. Splint placement did not stretch muscles on the front of the lower leg: the red and white portion of tibialis anterior and extensor digitorum longus muscle. The right hindlimb served as a non-stretched contralateral limb. For daily stretching, the rat was anaesthetized briefly (<1 min) with 1.5% isoflurane when the splint was secured to the left ankle using tape. During 30 min of muscle stretching, the rat was awake but immobilized in an acrylic restrainer. After stretching, the splint was taken off without anaesthesia. Sham control rats were briefly anaesthetized and immobilized in the restrainer for 30 min without a splint. Vascular responses, skeletal muscle blood flow measurements and harvesting of tissue for immunohistochemistry were performed at least 48 h after the last bout of muscle stretching.

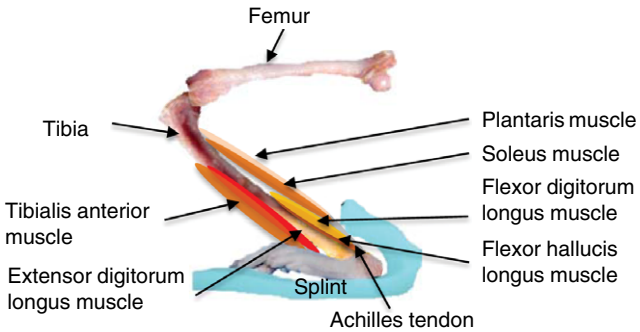

Figure 1. Muscle stretch using splint
An ankle dorsiflexion splint was applied to hold the left ankle joint at 30° of dorsiflexion. The ankle dorsiflexion splint elongated ankle plantar flexor muscles (soleus, plantaris, flexor hallucis longus and flexor digitorum longus) and shortened ankle dorsiflexor muscles (tibialis anterior and extensor digitorum longus).

Surgical preparation for determination of blood flow

Rats were anaesthetized with 2.5% isoflurane and a catheter filled with heparinized saline was advanced into the right carotid artery to the aortic arch. The carotid catheter was externalized at the base of the neck and secured to the skin between the shoulder blades. A second catheter, placed in the caudal tail artery and externalized at the base of the tail, was used to monitor mean arterial pressure and to obtain a reference blood sample for calculating tissue flows. After closure of the incisions, the animals were given ≥4 h to recover because previous studies demonstrated that circulatory dynamics, regional blood flow, arterial blood gases and acid–base status are stable in the awake rat 1–6 h after gas anaesthesia (Flaim *et al.* 1984).

Skeletal muscle blood flow at rest and during treadmill exercise

At the end of the recovery period, the rat was placed on the treadmill and exercise was initiated (15 m min^{-1} at a 0° incline). After 3 min of total exercise time, blood withdrawal was begun from the caudal tail artery at 0.25 mL min^{-1}. Radiolabelled microspheres were then infused into the carotid artery catheter (either ^{57}Co and ^{85}Sr, infused in random order; diameter 15 μm; ~2.5 × 10^5 in number), as described previously (Flaim *et al.* 1984; Delp *et al.* 1998; Musch *et al.* 2004; Behnke *et al.* 2012). Blood withdrawal from the caudal tail artery continued for 45 s after microsphere infusion was complete. After a 30 min recovery period from exercise, infusion of the second microsphere (either ^{57}Co and ^{85}Sr) was performed to measure resting blood flow. After the second microsphere infusion, the rat was killed with pentobarbital sodium (>100 mg kg^{-1} I.P.). Prior to blood flow evaluations, rats were familiarized with the treadmill exercise during three sessions of walking (10 min day^{-1} at 15 m min^{-1}, 0° incline).

Skeletal muscle blood flow during and after acute muscle stretching

Naïve rats (20 months old, male Fischer 344; $n = 17$) that did not undergo daily stretching via splint placement were used to determine the effects of acute splint placement (acute stretching). Naïve rats were acclimated to the splints and restrainers for 10 min day^{-1}, on three consecutive days. At least 48 h elapsed between the last acclimation session and blood flow assessment during/after acute splint placement (acute stretching). Catheters were implanted as described above.

After a 4 h recovery period, the rat was placed in the restrainer and radiolabelled microspheres (either ^{57}Co and ^{85}Sr, infused in random order) were infused for determination of resting blood flow. In one subgroup of rats, skeletal muscle blood flow was measured during muscle stretching. After measuring resting blood flow, the rat was taken out of the restrainer, briefly anaesthetized with 1.5% isoflurane when the splint was placed on left ankle, and then returned to the restrainer. Ten minutes after splint placement, infusion of the second microsphere (either ^{57}Co and ^{85}Sr) was performed. In another subgroup of rats, skeletal muscle blood flow was measured immediately after acute muscle stretching. After measuring resting blood flow, the rat was taken out of the restrainer, briefly anaesthetized with 1.5% isoflurane when the splint was placed on left ankle, and then returned to the restrainer. The rat remained in the restrainer with the splint in place for 30 min. A second microsphere (either ^{57}Co and ^{85}Sr) was infused into the carotid catheter ∼30 s after the splint was removed. After the second microsphere infusion, the rat was killed with pentobarbital sodium.

Blood flow analysis

Plantar flexor muscles (soleus, plantaris, flexor digitorum longus and flexor hallucis longus) and dorsiflexor muscles (red and white portion of tibialis anterior and the extensor digitorum longus) were dissected from stretched and non-stretched contralateral limbs and weighed. The radioactivity level of the individual muscles was determined using a gamma scintillation counter. Blood withdrawals from the caudal tail artery were utilized as reference samples. Blood flow to each muscle was calculated by reference sample method and expressed in mL min^{-1} 100 g tissue^{-1} according to the formula:

Blood flow (mL min^{-1} 100 g^{-1}) = [(gamma counter counts of the tissue/gamma counter counts of reference sample) × 0.25 mL min^{-1}]/[tissue wet weight (g)/100]

Kidney blood flows were used as an indicator of adequate mixing of microspheres; blood flow values were only considered valid if left and right kidney flows were within 15% of each other (Delp *et al.* 1998).

Microvessel preparation

Rats were weighed and anaesthetized with 3% isoflurane and killed by excision of the heart. The soleus-plantaris-gastrocnemius muscle group was removed from both the stretched and non-stretched contralateral hindlimb, and immediately placed in cold (4°C) filtered physiological saline solution (PSS). First-order arterioles were isolated from the soleus muscle and cannulated on pipettes and pressurized to 70 cmH_2O in an organ chamber that contained warm (37°C) PSS. The chamber was then placed on an inverted microscope equipped with a video camera and micrometer to measure intraluminal diameter. Soleus muscle arterioles without leaks were allowed to equilibrate for ∼ 1 h until developing ≥20% spontaneous tone. At the end of all experiments, arterioles were placed in Ca^{2+}-free PSS with 100 μM of sodium nitroprusside to determine the maximal diameter. Development of spontaneous tone was expressed as percentage constriction relative to maximal diameter and calculated as:

Spontaneous tone (%) = $(D_{max} - D_s)/D_{max} \times 100$ where D_{max} is the maximal inner diameter recorded at a pressure of 70 cmH_2O under Ca^{2+}-free conditions and D_s is the steady-tone baseline diameter.

Evaluation of vasodilatory responsiveness

Vasodilatory responses to cumulative addition of the endothelium-dependent vasodilator ACh (1×10^{-9} to 1×10^{-4} M) and the nitric oxide (NO) donor diethylamineNONOate (Dea-NONOate) (1×10^{-9} to 1×10^{-4} M) were determined in soleus muscle arterioles, as described previously (Muller-Delp *et al.* 2002).

Vasodilatory responses were expressed as percentage relaxation as calculated by the formula:

Relaxation (%) = $[(D_s - D_b)/(D_{max} - D_b)] \times 100$ where D_b is the steady baseline diameter before adding the first dose of the specific vasodilators, D is the steady diameter after addition of each dose of the vasodilators and D_{max} is the maximal inner diameter recorded under Ca^{2+}-free conditions.

Immunohistochemical analysis of arteriolar proteins

Protein levels of eNOS and superoxide dismutase (SOD) were assessed in soleus muscle arterioles isolated from stretched and non-stretched contralateral limbs. Arterioles were cannulated and pressurized to 70 cmH_2O. The arterioles were incubated at 37°C in Ca^{2+}-free PSS with 100 μM sodium nitroprusside for 1 h, fixed in 50% Bouin's solution and frozen in OCT. Cross-sections (5 μm) were cut on a cryostat. Sections were washed with PBS before adding a blocking solution of 0.3% Triton-X and 10% normal donkey serum at room temperature for 1 h. Sections were then incubated with primary antibodies against either eNOS (anti-eNOS antibody, dilution 1:200; Sigma, St Louis, MO, USA) or SOD (anti-superoxide dismutase 1 antibody, dilution 1:50; Abcam, Cambridge, MA, USA) at 4°C overnight. After PBS washes, species-specific anti-IgG (FITC; Abcam) was added at a dilution of 1:100 for 1 h at room temperature. After washing, 4′,6-diamidino-2-phenylindole (DAPI) was added and images were obtained using a fluorescence microscope. To exclude adventitial staining, a region of interest was established manually using ImageJ (NIH, Bethesda, MD, USA) after isolating the images for green

fluorescence. For each section, the average pixel intensity in the region of interest was obtained. Background was determined by incubating sections in the absence of primary and secondary antibodies and then subtracted from positively stained images. The average of the pixel intensity values obtained from at least three adjacent sections (with a coefficient of variation $\leq$0.25) was calculated and used for statistical analysis.

Immunohistochemical analysis of skeletal muscle angiogenesis

Rats were weighed and anaesthetized with 2.5% isoflurane and killed by excision of the heart. A laparotomy was performed to isolate the abdominal aorta from the vena cava, and a catheter filled with warm, heparinized papaverine solution (37°C, 1000 U mL^{-1} heparin in 0.9% saline, 4 mg L^{-1} papaverine) was advanced into the abdominal aorta. The vena cava was nicked and the heparinized papaverine solution was infused into the abdominal aorta at a rate of 0.05 mL s^{-1}. After clear saline emerged from the opening in the vena cava, 3.5 mL of saline containing 2% paraformaldehyde was infused into the abdominal aorta at the same rate. Soleus muscles from both stretched and non-stretched contralateral limbs were dissected, weighed and frozen in OCT compound. Eight-micron sections were cut on a cryostat. Sections were washed with PBS and incubated with rhodamine-labelled *Griffonia simplicifolia* lectin I (15 μg mL^{-1} diluted with PBS; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature in the dark (Hansen-Smith *et al.* 1988). Sections were rinsed with PBS, visualized with a fluorescence microscope at 100× magnification and images were captured with a digital charge-coupled device (CCD) camera. Muscle fibres were outlined by hand and colour and size thresholding was applied in MATLAB (MathWorks Inc., Natick, MA, USA) within the circumscribed area. The number of capillaries per field (one muscle fibre) was calculated automatically. The number of capillaries surrounding a muscle fibre was calculated for a minimum of five fields (fibres) per cross-section, and at least three adjacent sections were analysed per muscle. Capillary-to-fibre ratio was determined by counting capillaries and fibres contained within a static grid overlaid on images with identical area, as described previously (Mathieu-Costello *et al.* 1989). Image J was used to prevent multiple countings of fibres or capillaries.

To determine expression of angiogenic proteins, muscle sections were rinsed with PBS, incubated with blocking solution for 1 h at room temperature, and then incubated with primary antibodies [anti-hypoxia-induced factor-1α (HIF-1α), Abcam, dilution 1:100; anti-VEGFA, Abcam, dilution 1:100; anti-neuronal nitric oxide synthase (nNOS), Abcam, dilution 1:50] overnight at 4°C. After washing, secondary antibodies (Goat anti-Rabbit IgG H & L; Abcam) were added at a dilution of 1:1000 for 1 h at room temperature. After washing with PBS, DAPI was applied, and images were visualized with a fluorescence microscope at 100× magnification, and captured with a digital CCD camera. Background was determined by incubating sections in the absence of primary and secondary antibodies, and was subtracted from positively stained images. Three adjacent sections were used for image analysis. Three skeletal muscle cells were randomly selected and imaged from each section, and the average pixel intensity was determined for the region of interest defined by outlining the entire skeletal muscle cell. The average of the pixel intensity values obtained from at least three adjacent sections (with a coefficient of variation $\leq$ 0.25) was calculated and used for statistical analysis.

Micro-computed tomography (CT) scanning

Rats were weighed and anaesthetized with 2.5% isoflurane and killed by excision of the heart. A laparotomy was performed to isolate the abdominal aorta from the vena cava, and warm (37°C) heparinized papaverine solution (1000 U mL^{-1} of heparin in 0.9% saline, 4 mg L^{-1} papaverine) was infused into the abdominal aorta at a rate of 0.05 mL s^{-1}. After clear saline emerged from the opening in the vena cava, 3.5 mL of warm contrast medium (40°C, Microfil®; Flow Tech, Carver, MA, USA) was infused at the same rate. After yellow contrast medium emerged from the opening in the vena cava, the rat was placed in refrigerator at 4°C overnight to allow polymerization. The next day, the soleus muscle was dissected from both stretched and non-stretched contralateral limbs, weighed and saved in 2% paraformaldehyde. The soleus muscle vasculature was imaged using a high-resolution (6 μm voxel size) micro-CT imaging system (Scanco Medical, Basserdorf, Switzerland). Noise was eliminated using a low-pass Gaussian filter. All vertical long axis tomograms were thresholded to render binarized 3D images of the vascular network separated from the surrounding tissues. Soleus muscles from stretched and non-stretched contralateral limbs were evaluated individually to quantify 3D histomorphometric values, including absolute vascular volume, normalized vascular volume (vascular volume per unit muscle volume) and vessel connectivity (number of vascular bifurcations per unit muscle volume). To normalize vascular volume and connectivity, muscle volume was obtained by circumscribing the soleus muscle external margin and applying thresholding to the entire muscle 3D image.

Statistical analysis

Vessel responses to ACh and Dea-NONOate were evaluated using two-way ANOVA (stretch and dose/time)

with repeated measurement (dose/time). Group differences in animal characteristics, vessel characteristics, muscle blood flow, capillarity, and protein levels in arterioles and muscles were assessed by a paired *t* test or a Wilcoxon signed-rank test. In all data, the number of animals is indicated by '*n*'. $P < 0.05$ was considered statistically significant. All data are presented as the mean ± SE.

Results

Animals

Body weight and central haemodynamics (blood pressure and heart rate) for the groups are shown in Table 1. No differences between groups were observed for body mass or blood pressure at rest or during splinting (Table 1). Both heart rate and blood pressure were significantly elevated during exercise compared to rest (Table 1). Blood pressure was not altered during acute muscle stretching or immediately after acute muscle stretching compared to blood pressure at rest (Table 1). Body weight and blood pressure were not different in sham control rats compared to rats in which one leg had undergone daily stretching.

Vessel and muscle characteristics

The characteristics of skeletal muscles and arterioles are shown in Table 2. Wall thickness, spontaneous tone and maximal diameter of soleus muscle arterioles were not altered by muscle stretching. Soleus and plantaris muscle weights were significantly higher in the stretched compared to the non-stretched contralateral limb ($P < 0.01$, respectively) (Table 2); however, the weights of the flexor hallucis longus, flexor digitorum longus, and red and white portions of tibialis anterior and extensor digitorum longus muscles were not different between the stretched and the non-stretched contralateral limb.

Blood flow at rest and during treadmill exercise

After 4 weeks of muscle stretching, resting blood flow to the soleus, plantaris, flexor hallucis longus, flexor digitorum longus, red and white portion of tibialis anterior, and extensor digitorum longus muscles was not different between the stretched and non-stretched contralateral limbs (Fig. 2*A*). During treadmill exercise, blood flow to the soleus, plantaris, flexor hallucis longus and flexor digitorum longus muscles was significantly higher in the stretched limb compared to the non-stretched contralateral limb ($P < 0.05$, respectively) (Fig. 2*B*). Blood flow to the red and white portion of tibialis anterior and extensor digitorum longus muscles of the stretched and non-stretched contralateral limbs was not different during treadmill exercise.

Blood flow during and immediately after acute muscle stretching

During acute muscle stretching, blood flow to the soleus, plantaris, flexor hallucis longus and flexor digitorum longus muscles was significantly lower in the stretched limb compared to the non-stretched contralateral limb

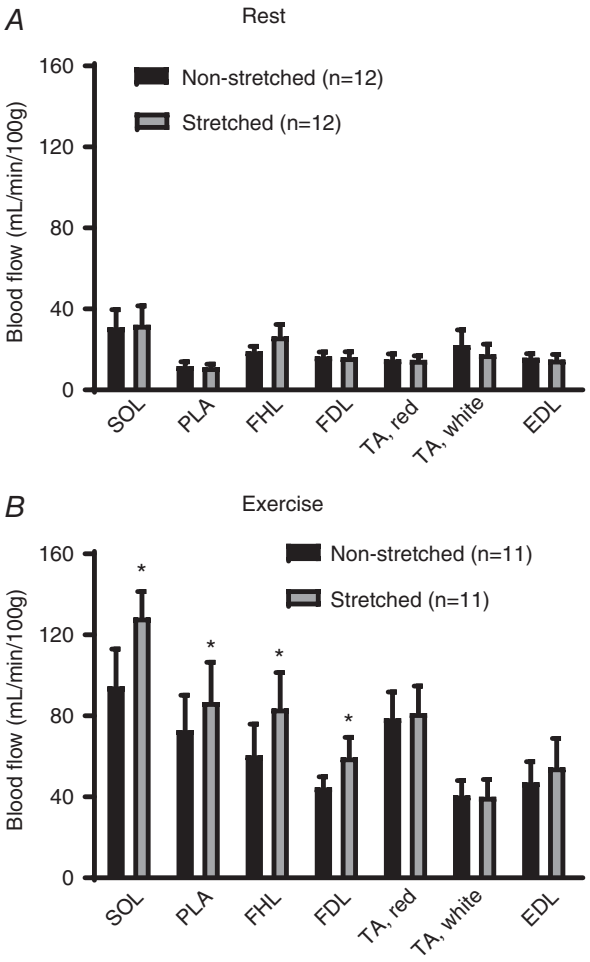

Figure 2. Blood flow at rest and during exercise after 4 weeks of muscle stretching
A, blood flow at rest was not different between any muscles from the stretched (grey) and non-stretched contralateral limbs (black). *B*, blood flow to SOL, PLA, FDL and FHL during treadmill exercise was higher in the stretched limb (grey) compared to the non-stretched contralateral limb (black); however, blood flow to TA and EDL was not different between the stretched and non-stretched contralateral limb. SOL, soleus muscle; PLA, plantaris muscle; FDL, flexor digitorum longus muscle; FHL, flexor hallucis longus muscle; TA red, red portion of tibialis anterior muscle; TA white, white portion of tibialis anterior muscle; EDL, extensor digitorum longus muscle; *n*, number of rats. *$P < 0.05$ *vs*. non-stretched contralateral limb. Values are the mean ± SE.

Table 1. Body weight and central haemodynamics

	Sham control ($n = 16$)	Stretched ($n = 35$)
BW (g)	463 ± 5	454 ± 8
SOL weight (mg)		
Non-stretched contralateral	178 ± 7	166 ± 4
Stretched	178 ± 6	184 ± 6*
SOL weight/BW (mg g^{-1})		
Non-stretched contralateral	0.38 ± 0.02	0.39 ± 0.01
Stretched	0.39 ± 0.02	0.43 ± 0.01*†
MAP (mmHg)		
At rest	136 ± 5	137 ± 3
During treadmill exercise	145 ± 5	148 ± 3
HR (beats min^{-1})		
At rest	346 ± 9	355 ± 9
During treadmill exercise	394 ± 11	401 ± 10
	Naïve rats ($n = 17$)	
MAP (mmHg)		
At rest	140 ± 3	
During acute muscle stretching	130 ± 4	
Immediately after acute muscle stretching	133 ± 2	
HR (beats min^{-1})		
At rest	350 ± 11	
During acute muscle stretching	352 ± 13	
Immediately after acute muscle stretching	366 ± 12	

BW, body weight; SOL, soleus muscle; MAP, mean arterial pressure; HR, heart rate. *$P < 0.05$ *vs.* non-stretched contralateral. †$P < 0.05$ *vs.* sham control; *n*, number of rats. Data are the mean ± SE.

($P < 0.05$, respectively) (Fig. 3*A*); however, no differences in blood flow were observed between the red and white portion of tibialis anterior and extensor digitorum longus muscle of the stretched and the non-stretched contralateral limb (Fig. 3*A*). Immediately after acute muscle stretching, blood flow to skeletal muscle was not different between any muscles of the stretched and the non-stretched contralateral limb (Fig. 3*B*). Muscle blood flow and blood pressure were evaluated in rats placed in the restrainer without splint placement and did not differ significantly from resting values (data not shown).

Vascular reactivity

Vasodilatory responses to ACh were significantly greater in skeletal muscle arterioles from the stretched limb compared to those from the non-stretched contralateral limb ($P < 0.001$) (Fig. 4*A*). ACh-induced vasodilatation of soleus muscle arterioles from cage control and sham control rats was not different, as shown in Fig. 4*A* (sham control). ACh-induced vasodilatation was significantly higher in soleus muscle arterioles from the stretched limb compared to those from the non-stretched contralateral limb, as well as those from cage and sham control rats ($P < 0.001$) (Fig. 4*A*). Inhibition of eNOS with L-NAME dramatically reduced ACh-induced vasodilatation of soleus muscle arterioles and eliminated differences in responsiveness between groups (Fig. 4*B*). Vasodilatory responses of soleus muscle arterioles to Dea-NONOate were not altered by muscle stretching (Fig. 4*C*).

Arteriolar protein levels

Figure 5*A* shows representative images of skeletal muscle arterioles stained with primary anti-eNOS or anti-SOD. Average pixel intensity of eNOS was higher in arterioles from the stretched limb compared to those from the non-stretched contralateral limb ($P < 0.05$) (Fig. 5*B*); however, SOD was not different between arterioles from the stretched and non-stretched contralateral limb.

Capillarity

Figure 6*A* shows representative images of soleus muscle stained with *G. simplicifolia* lectin I, anti-HIF-1α, anti-VEGFA or anti-nNOS. The number of capillaries surrounding each skeletal muscle fibre was significantly higher in the soleus muscle from the stretched limb compared to that from the non-stretched contralateral limb ($P < 0.05$) (Fig. 6*B*). The capillary-to fibre ratio was also increased in the soleus muscle from the stretched limb compared to that from the non-stretched contralateral limb (Table 2). Levels of HIF-1α and VEGFA were

Table 2. Vessel and muscle characteristics

	Non-stretched ($n = 35$)	Stretched ($n = 35$)	*P* value
Vessel wall thickness (μm)	20 ± 1	21 ± 1	0.298
Vessel tone (%)	68 ± 4	69 ± 3	0.203
Vessel diameter (baseline) (μm)	82 ± 6	74 ± 4	0.348
Maximum diameter (μm)	120 ± 5	115 ± 7	0.246
SOL weight (mg)	166 ± 4	184 ± 6	<0.001
SOL weight/BW (mg g^{-1})	0.39 ± 0.01	0.43 ± 0.01	<0.001
PLA weight (mg g^{-1})	336 ± 13	362 ± 14	0.007
PLA weight/BW (mg g^{-1})	0.78 ± 0.04	0.89 ± 0.04	0.005
FDL weight (mg)	149 ± 23	176 ± 25	0.077
FDL weight/BW (mg g^{-1})	0.36 ± 0.06	0.41 ± 0.06	0.133
FHL weight (mg)	73 ± 10	75 ± 9	0.431
FHL weight/BW (mg g^{-1})	0.14 ± 0.02	0.16 ± 0.03	0.136
TA weight (mg)	338 ± 38	344 ± 32	0.452
TA weight/BW (mg g^{-1})	0.79 ± 0.09	0.79 ± 0.08	0.475
EDL weight (mg)	162 ± 8	168 ± 14	0.375
EDL weight/BW (mg g^{-1})	0.39 ± 0.03	0.37 ± 0.02	0.288
Capillary to fibre ratio	2.31 ± 0.09	3.08 ± 0.04	<0.001

BW, body weight; SOL, soleus muscle; PLA, plantaris muscle; FDL, flexor digitorum longus muscle; FHL, flexor hallucis longus muscle; TA red, red portion of tibialis anterior muscle; TA white, white portion of tibialis anterior muscle; EDL, extensor digitorum longus muscle; *n*, number of rats. Data are the mean ± SE.

significantly higher in the soleus muscle from the stretched limb compared to the soleus muscle of the non-stretched contralateral limb ($P < 0.05$) (Fig. 6*B*). The level of nNOS was not different between the soleus muscle of the stretched limb and the soleus muscle of the non-stretched contralateral limb.

Muscle vascularity

Micro-CT analysis (Fig. 7*A*) revealed that vascular volume (normalized to total muscle volume) increased in the soleus of the stretched limb compared to that of the non-stretched contralateral limb (Fig. 7*B*). Absolute vessel volume and vascular connectivity tended to increase ($P = 0.098$ and 0.056, respectively), although the increase did not reach statistical significance (Fig. 7*B*).

Discussion

The main findings of the present study are that 4 weeks of daily muscle stretching (i) increased blood flow to skeletal muscle during exercise; (ii) enhanced endothelium-dependent vasodilatation of resistance arterioles; and (iii) increased several morphological indices of O_2 delivery capacity in stretched muscle of old rats. To our knowledge, this is the first report of the effect of daily muscle stretching on exercise hyperaemia in aged skeletal muscle. Our ankle dorsiflexion splint elongated ankle plantar flexor muscles and shortened dorsiflexor muscles; after 4 weeks of daily splint placement, exercise-induced hyperaemia in the muscles elongated during daily splint placement (soleus, plantaris, flexor hallucis longus and flexor digitorum longus muscles) was significantly augmented in the stretched limb. By contrast, blood flow during exercise was not altered in the tibialis anterior and the extensor digitorum longus, ankle dorsiflexor muscles which were shortened during daily splint placement. During acute muscle stretching, blood flow to the ankle plantar flexor muscles of the stretched limb was reduced by ∼60% compared to the same plantar flexor muscles of the non-stretched contralateral limb. By contrast, blood flow to dorsiflexor muscles of the stretched limb was not altered during acute muscle stretching. These results suggest that acute stretching produces a mechanical lengthening and local ischaemia within muscle, both of which may trigger vascular adaptations that contribute to increased blood flow during exercise in muscles that are stretched daily over 4 weeks.

Daily muscle stretching enhanced endothelium-dependent vasodilatation of soleus muscle arterioles (Fig. 4*A*). Additionally, eNOS protein levels were significantly higher in soleus muscle arterioles from the stretched limb compared to those from the non-stretched contralateral limb (Fig. 5*B*). Awolesi *et al.* (1995) reported increased eNOS in cultured endothelial cells after 24 h of cyclic stretch stimuli. Thacher *et al.* (2010) reported blunting of endothelium-dependent vasodilatation of isolated carotid arteries maintained without stretch for 24 h compared to 24 h of longitudinal cyclic stretch. In humans, Hotta *et al.* (2013) reported enhanced vascular endothelial

function after a single session of muscle stretching in patients with acute myocardial infarction. eNOS mRNA increases in vastus lateralis muscle of young male subjects after a single 90 min bout of passive knee movement (Hellsten *et al.* 2008) and after 4 weeks of passive knee movement for 90 min day^{-1}, 4 days week^{-1} (Hoier *et al.* 2010). Consistent with these reports, the results of the present study indicate that passive muscle stretching increases eNOS expression in intramuscular arterioles. By contrast

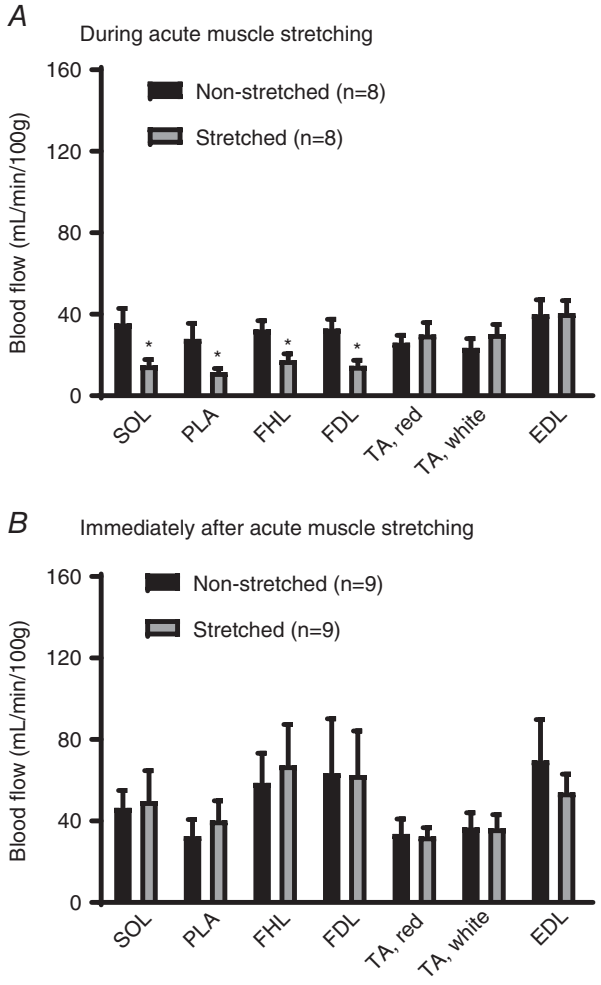

Figure 3. Skeletal muscle blood flow during and immediately after acute muscle stretching
A, blood flow to SOL, PLA, FDL and FHL during acute muscle stretching was lower in the stretched limb (grey) compared to the non-stretched contralateral limb (black); however, blood flow to TA and EDL was not different between the stretched and non-stretched contralateral limbs. *B*, skeletal muscle blood flow was not different between any muscles from the stretched and non-stretched contralateral limbs (*B*). SOL, soleus muscle; PLA, plantaris muscle; FDL, flexor digitorum longus muscle; FHL, flexor hallucis longus muscle; TA red, red portion of tibialis anterior muscle; TA white, white portion of tibialis anterior muscle; EDL, extensor digitorum longus muscle; *n*, number of rats; $^{*}P < 0.05$ *vs*. non-stretched contralateral limb. Values are the mean ± SE.

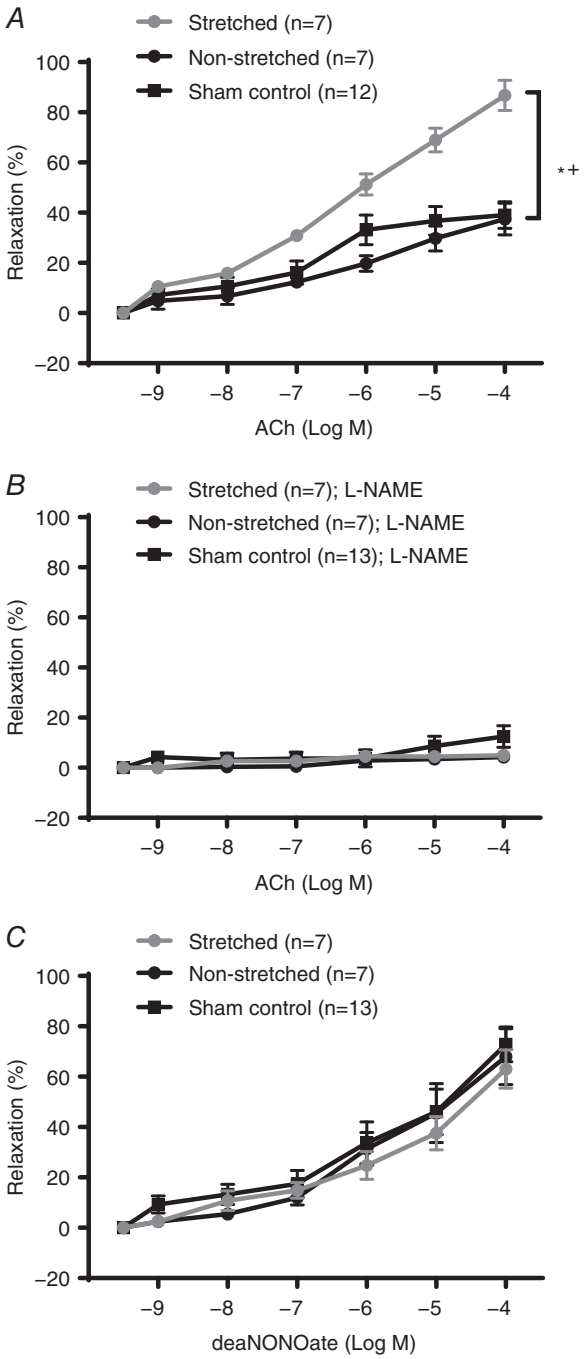

Figure 4. Endothelium-dependent and independent vasodilatation of soleus muscle arterioles
A, ACh-induced vasodilatation was significantly greater in soleus muscle arterioles from the stretched limb (grey) compared to dilatation of arterioles from the non-stretched contralateral limb (black) or limbs of sham control rats. *B*, inhibition with L-NAME eliminated differences in ACh-induced dilatation of soleus muscle arterioles from the stretched limb and the non-stretched contralateral limb. *C*, Dea-NONOate-induced vasodilatation was not different between soleus muscle arterioles from the stretched limb and the non-stretched contralateral limb stretch or limbs of sham control rats. *n*, number of rats; $^{*}P < 0.05$ *vs*. non-stretched contralateral limb; $^{+}P < 0.05$ vs. sham control.

to passive knee movements that increase muscle length at the same time as increasing muscle blood flow, our data indicate that static muscle stretching stimulates an increase in vascular eNOS expression without an increase in blood flow. By contrast, SOD levels were not changed by daily stretching, which is different from the increase in SOD content that occurs in muscle arterioles from old rats in response to treadmill exercise training (Sindler *et al.* 2013).

We also found that capillarity was higher in the soleus muscle of the stretched limb compared to the soleus muscle of the non-stretched contralateral limb, which is consistent with the notion that stretching-induced angiogenesis is a mechanism that contributes to enhanced exercise hyperaemia in the muscles of the stretched limb. Micro-CT analysis further indicated that stretching increased the number of microvascular connections and microvascular volume of the soleus muscle. Increased expression of HIF-1α, VEGF and angiogenesis can be triggered by several stimuli including mechanical forces (Holly *et al.* 1980; Milkiewicz *et al.* 2001; Rivilis *et al.*

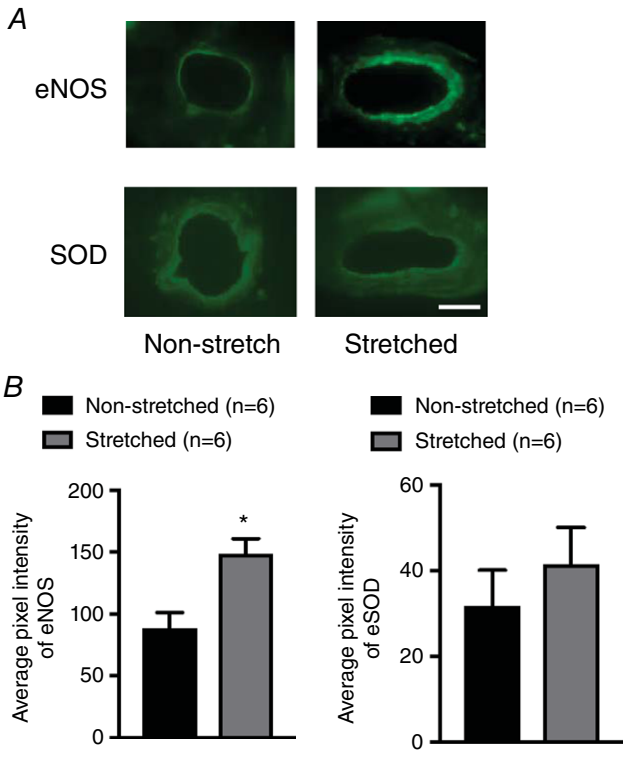

Figure 5. eNOS and SOD proteins in soleus muscle arterioles after 4 weeks of muscle stretching
A, representative images of soleus muscle arterioles from the stretched and non-stretch contralateral limbs. *B*, average pixel intensity of eNOS staining was higher in soleus muscle arterioles from the stretched limb. SOD staining in soleus muscle arterioles was not different between the stretched and the non-stretched contralateral limb. *n*, number of rats; $^*P < 0.05$ *vs.* non-stretched contralateral limb. Scale bar = 25 μm.

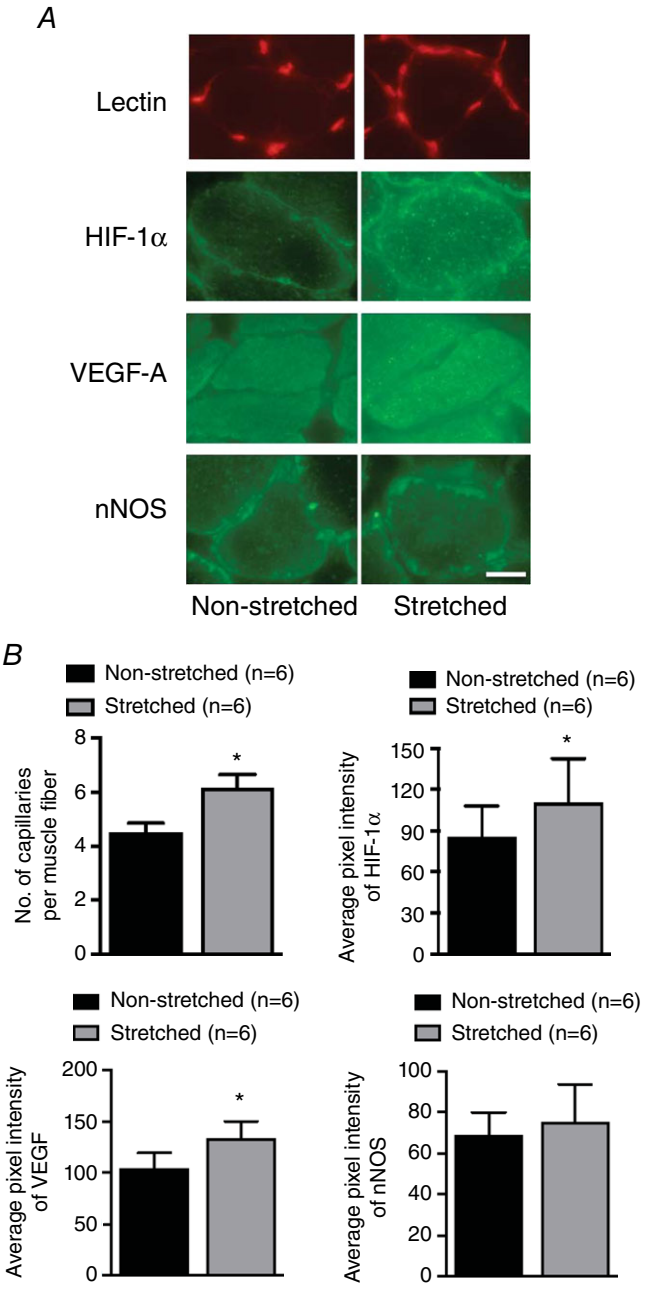

Figure 6. Capillarity and angiogenic proteins in soleus muscles after 4 weeks of muscle stretching
A, representative images of staining with lectin, HIF-1α, VEGF-A and nNOS in soleus muscles from stretched and non-stretched contralateral limbs. *B*, the number of capillaries per muscle fibre was higher in the soleus muscle from the stretched limb. Levels of HIF-1α and VEGF-A were higher in the soleus muscle from the stretched limb compared to the non-stretched contralateral limb; however, the level of nNOS was not different between the stretched limb and the non-stretched contralateral limb. *n*, number of rats; $^*P < 0.05$ *vs.* non-stretched contralateral limb. Scale bar = 25 μm.

2002), as well as exposure of vascular endothelial cells to a decreased P_{O_2} or ischaemia (Levy *et al.* 1995; Westvik *et al.* 2009). HIF-1α and VEGF both increase in skeletal muscle after a single bout of blood flow restricted exercise (Gustafsson *et al.* 1999) or with sustained stretch (Milkiewicz *et al.* 2007). Furthermore, there is abundant evidence to suggest that HIF-1A can promote the transcription of VEGFA (Forsythe *et al.* 1996) and

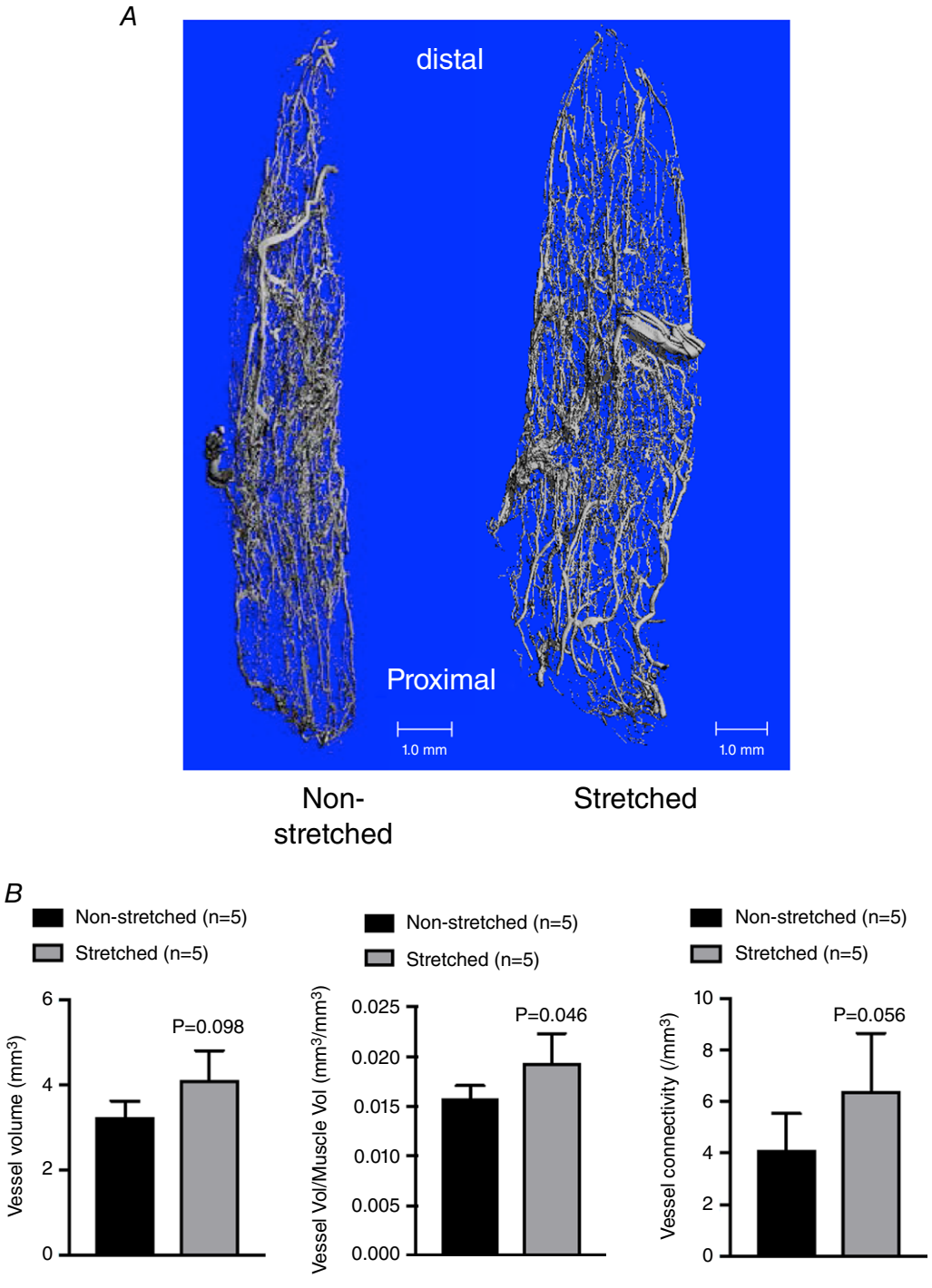

Figure 7. Micro-CT analysis of microvascular parameters after 4 weeks of muscle stretching
A, representative images of the soleus muscle microvasculature from the stretched and non-stretched contralateral hindlimb. *B*, absolute vessel volume (mm^3), normalized vascular volume (vascular volume/total muscle volume; mm^3/mm^3 and vessel connectivity in soleus muscles from stretched and non-stretched contralateral limbs. *n*, number of rats; $^*P < 0.05$ *vs*. non-stretched contralateral limb.

drive angiogenesis (Pajusola *et al.* 2005), although the precise mechanisms for the increased HIF-1α protein expression in the soleus observed in the present study are less clear. Consistent with reports of reduced skeletal muscle blood flow at extended sarcomere lengths (Poole *et al.* 1997; Kindig & Poole, 2001), we found that blood flow decreased during splint placement in muscles that were elongated by the dorsiflexion positioning of the ankle joint. According to the Fick principle, to maintain a given oxygen uptake in the face of reduced blood flow, there is an obligatory increase in muscle fractional O_2 extraction. Assuming that vascular diffusing capacity (D_{O_2}) is either unchanged or reduced, Fick's law dictates that a widening of the capillary-to-intramyocyte P_{O_2} gradient is requisite to increase extraction which, in the absence of an enhanced capillary or microvascular P_{O_2}, would require a lowering of intramyocyte P_{O_2} (Poole & Ferreira, 2007). However, during hypoxic gas breathing intracellular P_{O_2} values ($\sim$ 20 mmHg) (Richardson *et al.* 2006) are still four- to five-fold higher than that reported during even moderate intensity exercise ($\sim$5 mmHg) (Richardson *et al.* 2001). Thus, notwithstanding the intracellular hypoxia associated with reduced muscle blood flow during exercise, the reduction in blood flow observed during passive stretch in the soleus would probbaly not induce significant intracellular hypoxia. However, we cannot rule out local areas of ischaemia and hypoxia contributing, in part, to an increased HIF-1α signalling. A more probable mechanism driving the upregulation of HIF-1α in the present study is the prolonged mechanical deformation of the blood vessels associated with sustained muscle stretch. Specifically, HIF-1α expression is increased with cyclical mechanical stretch in vascular smooth muscle cells (Chang *et al.* 2003) and prolonged stretch in isolated vessels (Lim *et al.* 2011). Vascular endothelial cells sense extracellular mechanical stimuli such as shear stress and stretch and then transduce these signals into intracellular signalling cascades that trigger angiogenesis even under nonhypoxic conditions (Holly *et al.* 1980; Hoier *et al.* 2010). Thus, based upon current evidence, the repeated prolonged vascular deformation and stretch in muscles of the splinted limb is probably the main mechanism for the upregulated HIF-1α and VEGF *vs.* hypoxia, *per se*.

The involvement of nNOS in the angiogenic response to exercise training in skeletal muscle has been demonstrated previously (Huber-Abel *et al.* 2012). Tidball *et al.* (1998) reported that nNOS levels in skeletal muscle decreased after 10 days of hindlimb unloading, and returned to baseline levels after 2 days of reloading. Gavin *et al.* (2000) investigated the link between NO and angiogenic proteins using systemic NOS inhibition and found that NOS inhibition attenuates any exercise-induced increase of VEGF mRNA in rat skeletal muscle. Similarly, in humans, exercise training increases nNOS mRNA and protein, which is correlated with angiogenesis in vastus lateralis muscle (Huber-Abel *et al.* 2012). By contrast to these results but consistent with results reported by Williams *et al.* (2006), in which angiogenesis induced by unilateral extirpation was unaffected by deletion of nNOS, the results of the present study showed no difference in the level of nNOS between soleus muscles of the stretched and non-stretched contralateral limb.

A key mechanism for the old age-related reduction in exercise capacity is an oxygen delivery-to-demand mismatching (Behnke *et al.* 2005) resulting from alterations in skeletal muscle blood flow. The most common and arguably efficacious paradigm used to improve exercising muscle hyperaemia with old age is strenuous aerobic exercise training, which improves blood flow distribution during exercise (Behnke *et al.* 2012). The findings of the present study provide a novel method (via daily dorsiflexion splinting) to improve exercise hyperaemia in aged skeletal muscle. There are several important similarities between the effects of daily muscle stretching and those associated with chronic aerobic exercise training in old age. First, neither daily muscle stretching (Fig. 2*A*), nor exercise training (Behnke *et al.* 2012) affected skeletal muscle blood flow at rest. Second, both daily muscle stretching (Fig. 2*A*) and aerobic exercise training (Behnke *et al.* 2012) increased skeletal muscle blood flow during moderate intensity exercise. Importantly, the increase in exercise hyperaemia between the two paradigms was quantitatively similar ($\sim$30% increase in blood flow during exercise) with daily muscle stretching (Fig. 2*B*) and aerobic exercise training (Behnke *et al.* 2012). Third, daily muscle stretching increased muscle capillarity (Fig. 6) and VEGF expression (Fig. 6) similar to that occurring in aged skeletal muscle after exercise training (Gavin *et al.* 2015). An important difference between the two paradigms is that the beneficial effects of daily stretching were confined to muscles displaying an ischaemic response during stretch (Fig. 3*A*), whereas aerobic exercise training can induce systemic cardiovascular improvements (McCullough *et al.* 2011). We would not expect systemic cardiovascular benefits, nor local improvements in the oxidative capacity of the muscle as observed with chronic aerobic exercise training (Spier *et al.* 2004); however, we did not measure muscle oxidative capacity. It is possible that oxidative capacity was reduced in the muscles demonstrating an increased expression of HIF-1α because this protein has been implicated in the suppression of mitochondrial biogenesis (Mason *et al.* 2004).

In summary, the results of the present study suggest that daily muscle stretching induces enhanced endothelium-dependent vasodilatation and angiogenesis, enhancing exercise-induced hyperaemia in the skeletal muscles of aged rats. Local ischaemia and/or mechanical stretching of intramuscular blood vessels are probable triggers of these vascular adaptations in chronically stretched skeletal muscle. Thus, a programme of passive

muscle stretching, in the absence of pharmacological or surgical intervention, is sufficient to induce angiogenesis and augment exercise hyperaemia in aged skeletal muscle. Skeletal muscle blood flow is clinically important in elderly patients who have frailty, heart failure or peripheral artery disease. Muscle stretching performed with a splint could provide a feasible means of improving muscle blood flow and function in patients that cannot perform regular aerobic exercise. Clinical studies of the effects of muscle stretching on skeletal muscle blood flow in elderly patients are needed.

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Additional information

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

KH and JMD designed the study, including the muscle stretch using splints, blood flow measurements, vessel experiments and immunohistochemistry. KH, BA, MLE and JMD designed the micro-CT analysis. KH, BJB and JMD contributed to writing the manuscript. KH performed the statistical analyses and prepared the figures. KH, BJB, PM, DK and JMD contributed to the blood flow measurements. KH, PG, BC and JMD contributed to the vessel experiments. KH, PG, BC and RB contributed to daily muscle stretch training. KH, RB, MK and AC performed the immunohistochemistry. KH and JJM contributed to MATLAB pogramming and image analysis of immunohistochemistry. BJB, DDC and JMD revised the manuscript. KH, JMD and DDC obtained financial support. All authors checked and approved final version of manuscript submitted for publication.

Funding

This work was supported by National Institutes of Health Grants R21 AG-044858 (to Judy M. Muller-Delp and Demetra D. Christou) and The 27th Japan Heart Foundation/Bayer Yakuhin Research Grant Abroad (to Kazuki Hotta).