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Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training

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Non-technical summary Exercise training is a potent stimulus for capillary growth in skeletal muscle, but the precise mechanisms underlying the regulation of capillary growth in muscle remain unclear. We examined the effect of acute exercise and endurance training in male subjects, on a number of compounds believed to either promote or inhibit growth of capillaries in skeletal muscle. The results show that acute exercise increases the gene expression of both capillary growth-promoting and -inhibiting compounds, suggesting that both positive and negative factors are needed for the precise control of growth. Training increased capillary growth but had little effect on gene and protein levels of the capillary growth-promoting and -inhibiting factors, suggesting a similar potential for capillary growth in untrained and trained muscle. The study is one of the first addressing how the balance between a large number of positive and negative factors is affected in human muscle with exercise and training.

Abstract This study examined the effect of acute exercise and 4 weeks of aerobic training on skeletal muscle gene and protein expression of pro- and anti-angiogenic factors in 14 young male subjects. Training consisted of 60 min of cycling (~60% of $\dot{V}_{O_2max}$), 3 times/week. Biopsies were obtained from vastus lateralis muscle before and after training. Muscle interstitial fluid was collected during cycling at weeks 0 and 4. Training increased ($P < 0.05$) the capillary: fibre ratio and capillary density by 23% and 12%, respectively. The concentration of interstitial vascular endothelial growth factor (VEGF) in response to acute exercise increased similarly (>6-fold; $P < 0.05$) before and after training. Resting protein levels of soluble VEGF receptor-1 in interstitial fluid, and of VEGF, thrombospondin-1 (TSP-1) and tissue inhibitor of matrix metalloproteinase-1 (TIMP1) in muscle were unaffected by training, whereas endothelial nitric oxide synthase protein levels in muscle increased by 50% ($P < 0.05$). Before and after training, acute exercise induced a similar increase ($P < 0.05$) in the mRNA level of angiopoietin 2, matrix metalloproteinase 9 and TSP-1. After training, TIMP1 mRNA content increased with exercise ($P < 0.05$). In conclusion, acute exercise induced a similar increase in the gene-expression of both pro- and anti-angiogenic factors in untrained and trained muscle. We propose that the increase in anti-angiogenic factors with exercise is important for modulation of angiogenesis. The lack of effect of training on basal muscle VEGF protein levels and VEGF secretion during exercise suggests that increased VEGF levels are not a prerequisite for exercise-induced capillary growth in healthy muscle.

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Abbreviations Ang1, angiopoietin-1; Ang2, angiopoietin-2; BrdU, bromodeoxyuridine; eNOS, endothelial nitric oxide synthase; MMP2, matrix metalloproteinases-2; MMP9, matrix metalloproteinases-9; NO, nitric oxide; PF4, platelet factor-4; sVEGFR-1, soluble VEGF receptor-1; Tie2, angiopoietin receptor-2; TIMP1, tissue inhibitor of MMPs-1; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

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Introduction

Angiogenesis is regulated by a large number of pro- and anti-angiogenic factors where the balance between them is likely to determine if there will be growth or regression of capillaries (Egginton, 2009). The majority of data on the different pro- and anti-angiogenic factors stem from pathology whereas less is known about their role in skeletal muscle angiogenesis. The pro-angiogenic growth factor believed to be the most important in capillary growth in most tissues, including skeletal muscle, is vascular endothelial growth factor (VEGF) (Lloyd *et al.* 2003; Wagner *et al.* 2006; Olfert *et al.* 2010). In skeletal muscle, VEGF is present in endothelial cells as well as within skeletal muscle cells and upon contraction (Hoffner *et al.* 2003), or passive movement (Hoier *et al.* 2010), a fraction of the VEGF is secreted into the extracellular fluid where it may act on capillary endothelial cells.

An acute bout of exercise is known to induce an up-regulation of VEGF mRNA (Benoit *et al.* 1999; Jensen *et al.* 2004*b*) as well as VEGF protein (Ryan *et al.* 2006; Rullman *et al.* 2007) within the first hours after exercise. It could therefore be expected that VEGF protein levels are increased after a period of exercise training. In accordance, VEGF protein levels have been found to increase after endurance training of patients with cardiovascular disease (Gustafsson *et al.* 2001; Hansen *et al.* 2010) and in one study, increased VEGF levels were reported after only seven training sessions (Gustafsson *et al.* 2002). The absolute level of VEGF in muscle is likely to be relevant for the magnitude of VEGF secreted from muscle as it has been observed that the training-induced increase in muscle VEGF protein in hypertensive individuals is associated with an increased amount of interstitial VEGF after exercise (Hansen *et al.* 2010). However, these effects of training on VEGF may be related to the impairments in the VEGF system evident in the disease state, and it is not known whether there is a comparable association between training-induced alterations in muscle VEGF protein levels and secretion in young healthy individuals. One previous study has examined dialysate VEGF levels with training (Gavin *et al.* 2007); however, the general VEGf levels during acute exercise in the study by (Gavin *et al.* 2007), were several-fold lower than previously reported (Hoffner *et al.* 2003; Jensen *et al.* 2004*b*), probably due to a combination of very low probe-recovery and no determination of relative loss to estimate interstitial concentrations. Thus the VEGF data from Gavin *et al.* (2007) are associated with some uncertainty.

A limited number of studies in animals and humans have determined levels of angiogenic factors in skeletal muscle at the gene and protein level in association with acute exercise and training (Breen *et al.* 1996; Lloyd *et al.* 2003; Rullman *et al.* 2007), and few studies have addressed how the balance between different pro- and anti-angiogenic factors may be altered. Among the pro-angiogenic factors is nitric oxide (NO), formed by endothelial nitric oxide synthase (eNOS). Similar to VEGF, eNOS is up-regulated by shear stress (Williams *et al.* 2006*a*) and NO has been shown to regulate VEGF expression (Tsurumi *et al.* 1997). Other pro-angiogenic factors are matrix metalloproteinases (MMPs) and angiopoietin-2, which are important for the degradation of the extracellular matrix and capillary destabilization during sprouting angiogenesis (Rivilis *et al.* 2002). Anti-angiogenic factors include angiopoietin-1 (Ang1) which is important for stabilization of the newly formed capillary and which competes with Ang2 for the angiopoietin receptor-2 (Tie2). Tissue inhibitor matrix metalloproteinases (TIMPs) inhibit MMPs and thereby limit the extent of extracellular matrix degradation. Thrombospondin-1 (TSP-1) is a multifunctional protein with binding sites specific for cell-to-cell and cell-to-matrix interactions (Lawler, 1986). Deletion of TSP-1 in mice has been shown to increase capillarization in cardiac and skeletal muscle providing evidence for an important role for TSP-1 in restricting capillary growth (Malek & Olfert, 2009). Moreover, soluble VEGF receptor-1 (sVEGFR-1) binds and inactivates VEGF (Kendall & Thomas, 1993) and has been proposed to be an important regulator of VEGF signalling (Kendall *et al.* 1996). Finally, platelet factor-4 (PF4) is an inhibitor of angiogenesis that has been found to increase in plasma in response to acute exercise (Placanica *et al.* 1999); however, little data exist on the expression of PF4 in skeletal muscle in response to exercise.

The first hypothesis of the present study was that acute exercise performed before training would promote angiogenesis and increase the gene expression of pro-angiogenic factors and decrease the gene expression of anti-angiogenic factors. The second hypothesis was that endurance training would increase the transcriptional activity and thereby the protein level of pro-angiogenic factors and, in parallel, down-regulate protein levels of anti-angiogenic factors. Moreover, as significant capillary growth was expected to have occurred after the 4 weeks of training (Jensen *et al.* 2004*a*), we hypothesized that the need for angiogenesis was reduced after the training period and, therefore, the transient increase in mRNA after acute exercise would be reduced for pro-angiogenic factors and enhanced for anti-angiogenic factors.

Methods

Subjects

Fourteen healthy male subjects with a mean age of 31.5 (range 24–39) years, weight of 86.2 (range 66–116) kg, height of 181.7 (range 173–195) cm, and a pulmonary

maximal oxygen uptake ($\dot{V}_{O_2max}$) of 3.3 (range 2.7–4.2) l min^{-1} were included in the study. The subjects were non-smokers and habitually active but performed no regular training. The subjects were fully informed of the risks and discomfort associated with the study and all provided written consent. The study was carried out in accordance with the guidelines contained in the *Declaration of Helsinki* and was approved by the local ethics committee of Copenhagen.

Experimental design

Training was performed 3 times/week for 4 weeks and consisted of 60 min of continuous cycling on a cycle ergometer. The training was performed at ∼60% of $\dot{V}_{O_2max}$ determined prior to the training period for the first 2 weeks. Thereafter, the intensity was increased based on heart rate during a training session to ∼68% of $\dot{V}_{O_2max}$ determined prior to the training period. In total each subject performed 13 trainings sessions. At the first and last training session, an experiment was performed. The acute exercise bout was performed at the same absolute intensity on the two experimental days.

Experimental protocol

On the morning of the experimental day the subjects had a light breakfast. The subjects rested in the supine position, and the skin, subcutaneous tissue and fascia of the thigh were anaesthetized by injection with lidocaine (Xylocaine; 20 mg ml^{-1}) to prepare for muscle biopsy and insertion of microdialysis probes. A resting biopsy was then obtained from vastus lateralis muscle and either immediately frozen in liquid nitrogen or embedded in mounting medium and frozen in pre-cooled isopentane and stored at −80°C until further analysis. Microdialysis probes were placed in the thigh muscle as previously described (Hoffner *et al.* 2003) and perfused with buffer including a small amount (3.1 nM) of labelled adenosine [2-^{3}H] for calculation of probe recovery for each sampling (Hoffner *et al.* 2003). Approximately 50 min after probe insertion, the subjects performed 10 min of exercise at a power output of 10 W (Nordsborg *et al.* 2003). Approximately 90 min after insertion of the probes, dialysate was collected for two periods of 20 min while the subject was resting. Subsequently, 60 min of cycling at ∼60% of $\dot{V}_{O_2max}$ (144 ± 6 W) was carried out. Dialysate samples were collected throughout the exercise period. One hour and 3 h after exercise, additional biopsies were taken and treated the same way as the biopsy obtained at rest.

All dialysate samples were immediately frozen and stored at −80°C until time of analysis. Flow rate was calculated to estimate any loss of fluid or abnormal decrease in perfusion rate (Hoffner *et al.* 2003). Only probes with flow rates >4.0 μl min^{-1} and <6.0 μl min^{-1} were used for further analysis. The relative loss for each probe was determined according to the internal reference method (Scheller & Kolb, 1991; Jansson *et al.* 1994) for [2-^{3}H]adenosine. The relative loss of the probes was used to account for the difference in recovery from rest to exercise. We have found that although absolute recoveries can vary some between different compounds, the relative change in recovery is similar.

Immunohistochemistry

Transverse sections 8 μm in thickness were placed onto glass slides, fixed by immersion in acetone −20°C for 30 s and incubated for 2 min in 2% paraformaldehyde (pH 7.4) at room temperature. The sections were blocked for 1 h with PBS-1% BSA (pH 7.4). The muscle sections were incubated with primary antibody CD-31 (M0822, DakoCytomation, Denmark), diluted 1:10 in PBS-1% BSA for 1 h followed by incubation with biotinylated secondary antibody (ABComplex/AP KO376, Dako A/S, Denmark or AK-500, Vectastain; Vector Laboratories, Burlingame, USA) for 30 min. Binding was visualized with a Fuchsin + Substrate-Chromogen system (KO625, Dako A/S, Denmark). Specificity of the staining was assessed by staining without the primary antibody. Number of capillaries and fibres were determined on 160 ± 10 fibres per biopsy using light microscopy (Axiolab, Zeiss). Capillary supply was expressed as capillaries per fibre (C:F ratio), capillaries around a fibre (CAF) and capillary density (CD; cap. mm^{-2}). Mean fibre area was assessed by manual drawing of the perimeter of each muscle fibre using the image analysis computer software Tema (Version 95, Denmark).

Dialysate VEGF and sVEGFR-1 protein measurements

Dialysate VEGF and sVEGFR-1 protein levels were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (Quantikine Human VEGF and Human sVEGFR-1/Flt-1; R&D Systems, Minneapolis, MN, USA). The VEGF and sVEGFR-1 protein concentrations were measured in the dialysate and the interstitial concentrations were estimated by determination of the relative loss of tritium-labelled adenosine for each sample (Hoffner *et al.* 2003).

Measurement of endothelial cell proliferation

Human umbilical vein endothelial cells were used, supplemented with Medium 200, a low serum supplement intended for use in the culture of endothelial cells.

The supplement contained fetal bovine serum, fibroblast growth factor, heparin, and epidermal growth factor (Cytotech MK-200-2 Medium 200 kit). The endothelial cells were grown on 96-well plates for 24 h before the medium was replaced with 50 μl of microdialysate, perfusate, or supplemented Medium 200. None of the values exceeded the positive control (addition of Medium 200 with growth supplement). After an additional 30 h of incubation, bromodeoxyuridine (BrdU) was added and then incubated for 12 h. Incorporation of BrdU into the DNA was detected using an immunoassay (Roche, Mannheim, Germany) according to manufacturer's recommendations.

Western blot analysis

Biopsies (~30 mg) were freeze dried and dissected fat free prior to homogenization in a buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (Hepes), 1% nonyl phenoxylpoly-ethoxylethanol (NP-40), 20 mM β-glycerophosphate, 2 mM sodium orthovanadate (Na_3VO_4), 10 mM NaF, 2 mM phenylmethylsulphonyl-fluoride, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, and 3 mM benzamidine). Samples were rotated end over for 60 min at 4°C and then centrifuged for 30 min at 17,000 *g* at 4°C. The lysate was collected and protein concentration was determined by a BSA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Lysate proteins were separated on 16.5% or 7.5% Tris-Tricine gels (BioRad) and transferred semi-dry to PVDF membranes (Millipore AMC, USA). Human standard samples were loaded onto each gel for determination of a standard curve for VEGF and eNOS. The membranes were incubated with primary antibodies to VEGF (A-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), eNOS (610297 BD Transduction Laboratories), TSP-1 (ab85762, Abcam, USA), TIMP1 (AF 970, R&D Systems, UK), citrate synthase (ab96600, Abcam, USA). Secondary antibody horseradish-peroxidase-conjugated goat anti-rabbit (P-0448, Dako, Glostrup, Denmark) 1:5000 was used for detection of the proteins. Subsequent to exposure (Kodak Image Station, 2000MM) and quantification (Kodak Molecular Imaging software), the protein content was expressed in arbitrary units related to human standards.

Analysis of skeletal muscle mRNA content: RNA isolation, reverse transcription, and PCR

Total RNA was isolated from the muscle biopsies using TRIzol reagent according to the guidelines of the manufacturer (Invitrogen, CA, USA). First strand cDNA was synthesized from 1 μg total RNA by Super-Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as previously described (Pilegaard *et al.* 2000). The mRNA content of VEGF, eNOS, MMP9, MMP2, TIMP1, Ang2, Ang1, Tie2, TSP-1 and PF4 was determined by real-time PCR (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). The cDNAs were amplified using TaqMan Gene expression assays from Applied Biosystems. Prior optimization was performed as previously described (Pilegaard *et al.* 2003). For each sample, the amount of target gene mRNA was normalized to the cDNA content of the specific sample. cDNA amounts were determined by use of Oligreen as previously described (Lundby *et al.* 2005). The effect of the experimental conditions on the level of cDNA was statistically determined. No significant effect on mRNA content for the various proteins was found with acute exercise or training.

Statistics

All data are expressed as mean ± SEM. A one-way ANOVA with repeated measures was performed in order to evaluate the effect of training on capillarization, sVEGFR-1 protein concentration in dialysate, and muscle protein levels. A two-way ANOVA with repeated measures was performed in order to evaluate the effect of time and training on the VEGF protein concentration in dialysate, on measurements of the proliferative effect of muscle dialysate on endothelial cells, and on mRNA levels in the muscle biopsies. A Student-Newman-Keuls method for multiple comparisons was used to locate differences. A level of $P < 0.05$ was considered statistically significant.

Results

Capillarization

The capillary to fibre ratio (C:F) increased by 23% (2.47 ± 0.10 to 3.03 ± 0.16, $P < 0.001$) (Fig. 1*A*), number of capillaries around a fibre (CAF) was elevated 20% (4.25 ± 0.17 to 5.09 ± 0.22, $P < 0.001$) (Fig. 1*B*), and capillary density (CD) increased 12% (511 ± 19 to 571 ± 24, $P < 0.05$) (Fig. 1*C*) during the training period. Mean fibre area (μm^2) was not significantly altered during the training period (Fig. 1*D*).

Muscle interstitial VEGF protein

Before training, interstitial VEGF levels were increased 7-fold ($P < 0.001$) during acute exercise (from 141 ± 34 pg ml^{-1} at rest to 936 ± 200 pg ml^{-1}). After

training, exercise induced an increase in interstitial VEGF of a similar magnitude to that observed before training (Fig. 2).

Muscle interstitial sVEGFR-1 protein

The resting level of sVEGFR-1 protein remained unaltered with 4 weeks of training (Fig. 3).

Probe recovery

The relative recovery of the probes used for VEGF protein determination increased 24% (from 0.41 to 0.51; $P < 0.001$) from rest to exercise before training and 37% (from 0.47 to 0.64; $P < 0.001$) from rest to exercise after training.

Effect of muscle interstitial fluid on proliferation

Analysis of the proliferative effect of interstitial fluid on cultured endothelial cells by measurements of BrdU incorporation into cells, showed that dialysate obtained during exercise before and after training induced a ∼7- to 8-fold higher ($P < 0.001$) proliferative effect than did dialysate at rest (Fig. 4). The proliferative effect of the interstitial fluid obtained during exercise was unaffected by training.

Protein levels of angiogenic factors and citrate synthase

The basal content of VEGF protein as well as TSP-1 and TIMP1 protein in muscle was unaltered with 4 weeks of training (Fig. 5*A*, *C* and *D*). eNOS (Fig. 5*B*) and citrate synthase protein content increased 1.5-fold ($P < 0.05$) with training.

Muscle mRNA content of angiogenic factors

Before training the levels of VEGF, MMP9, Ang2, Ang2/Ang1 ratio, Tie2, and TSP-1 mRNA were higher ($P < 0.05$) after acute exercise compared to rest (Fig. 6*A*, *C*, *F*, *H*, *I* and *J*). After training MMP9, Ang2, and TSP-1 mRNA levels were elevated ($P < 0.05$) after acute exercise compared to rest (Fig. 6*C*, *F* and *J*). There was no effect of training on the levels of MMP9, Ang2, and TSP-1 mRNA after acute exercise.

Discussion

A main finding in the present study was a transient increase in the mRNA level of several pro- as well as anti-angiogenic factors in response to acute exercise and we propose that the increase in anti-angiogenic factors after exercise may be important for a well-controlled angiogenic process. In contrast to our hypothesis, the basal protein and mRNA

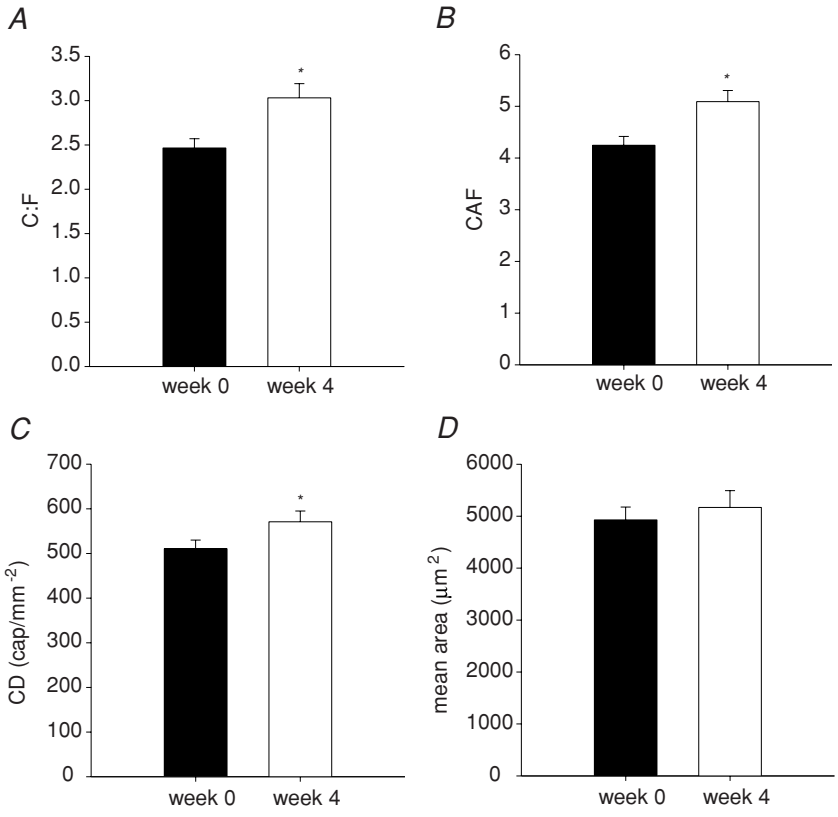

Figure 1. Capillarization in skeletal muscle cells before and after 4 weeks of moderate continuous cycle training
Capillary-to-fibre ratio (C:F, *A*), no. of capillaries around each fibre (CAF, *B*), capillary density (CD) (cap. mm^{-2}, *C*), and mean fibre area (μm^2, *D*) before (filled bars) and after 4 weeks of moderate continuous training (open bars). Values are mean ± SEM ($n = 14$). *$P < 0.05$ *vs.* week 0.

levels and the acute mRNA increases with exercise, for several pro- and anti-angiogenic factors, were not very different before and after training, suggesting a similar angiogenic stimulus in untrained and trained healthy muscle. This lack of effect of training was also true for VEGF as basal VEGF protein levels and exercise-induced increases in interstitial VEGF protein were analogous before and after training.

Based on observations of an up-regulation of VEGF mRNA (Benoit *et al.* 1999; Jensen *et al.* 2004*b*) as well as VEGF protein (Ryan *et al.* 2006; Rullman *et al.* 2007) in response to acute exercise, we postulated that exercise training would lead to an increased level of skeletal muscle VEGF protein. However, although the training was effective in increasing capillarization as well as a marker for mitochondrial capacity, citrate synthase, VEGF protein in muscle was not found to be enhanced after training. This finding could suggest that, in young healthy individuals, the existing VEGF stores in the muscle are sufficiently large and an increase is not required for the angiogenic response. This finding is in contrast to results from individuals with essential hypertension where 4 months of exercise training lead to a 40% increase in the skeletal muscle levels of VEGF (Hansen *et al.* 2010), as well as results from patients with cardiac heart failure showing enhanced VEGF protein levels after 8 weeks of aerobic training (Gustafsson *et al.* 2001). The discrepancy suggests that training mainly affects VEGF protein levels positively in individuals with reduced VEGF protein levels, e.g. in cardiovascular disease (Gustafsson *et al.* 2001; Hansen *et al.* 2010).

The amount of VEGF secreted from muscle to the interstitium upon contraction has been found to be related to the level of VEGF protein in the muscle (Hansen *et al.* 2010). In individuals with essential hypertension, lower VEGF protein levels in muscle were associated with a lack of VEGF secretion in response to acute exercise. However, when muscle VEGF protein levels were increased by a period of training, exercise did induce a significant secretion of VEGF (Hansen *et al.* 2010). An association between muscle VEGF protein levels and VEGF secretion is also supported by reports of lower muscle VEGF protein levels (Ryan *et al.* 2006) and lower dialysate VEGF protein levels (Gavin *et al.* 2007) in aged compared to young individuals. In the current study, the finding of unaltered VEGF protein levels before and after training, as well as a similar increase in the exercise-induced interstitial VEGF levels, would support such an association and suggests that an enhancement in muscle VEGF levels, or the amount secreted from muscle, is not a pre-requisite for angiogenesis in young healthy individuals. This latter observation fits with our notion that VEGF secretion from muscle is an all or nothing response, without apparent relationship between the amount of VEGF secreted and exercise intensity (Hoier *et al.* 2010).

One factor that could affect the concentration of VEGF in the interstitium which was not measured in the present study is VEGF clearance. We know from measurements of relative loss of radioactive tracers from the perfusate buffer in the microdialysis probes that the loss is increased with muscle contraction of tracer, indicating increased removal. It cannot be excluded that the training affected the rate of removal of VEGF from the interstitial space, thus affecting VEGF concentrations.

sVEGFR-1 binds to VEGF and neutralizes its effect and thereby acts as a negative modulator of angiogenesis (Kendall & Thomas, 1993; Kendall *et al.* 1996). We expected sVEGFR-1 to increase in the muscle dialysate after training to modulate the effect of interstitial VEGF to prevent excessive capillary growth. However, in contrast, the basal levels of sVEGFR-1 remained unaltered in the muscle interstitium after training compared to before, suggesting either that the angiogenic process was ongoing or that sVEGFR-1 may not be an important regulator of VEGF activity.

The level of eNOS protein was increased with training. eNOS has been implicated in angiogenesis, in part

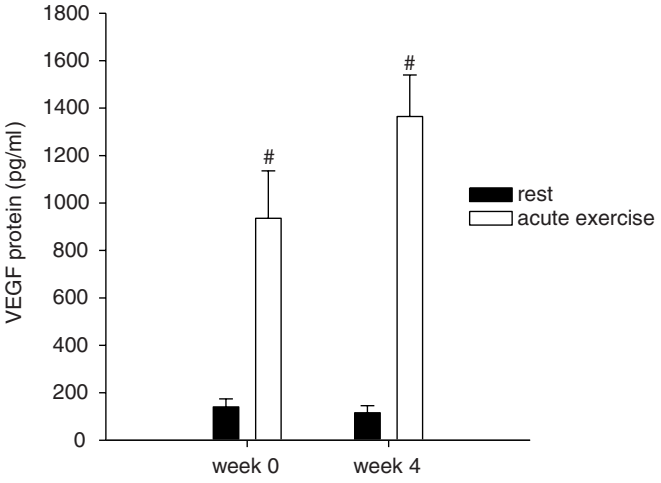

Figure 2. Interstitial concentration of VEGF protein in skeletal muscle at rest and during moderate continuous exercise before and after 4 weeks of moderate continuous cycle training
The concentration of VEGF protein was measured in the dialysate and the concentration in the interstitium was estimated by determination of relative loss of tritium-labelled adenosine for each probe. Microdialysate samples were collected at rest (filled bars) and during continuous exercise for 0–60 min (open bars). Values are means ± SEM ($n = 12$). #$P < 0.001$ *vs.* rest.

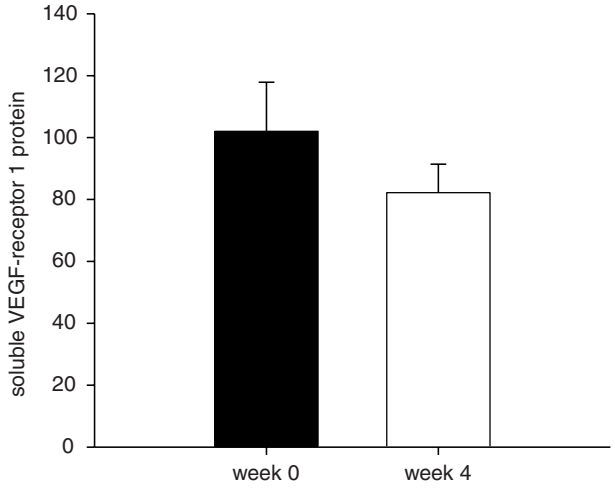

Figure 3. Interstitial concentration of soluble VEGF receptor-1 protein in skeletal muscle before and after 4 weeks of moderate continuous cycle training at rest
The concentration of sVEGFR-1 protein was measured in the dialysate and the concentration in the interstitium was estimated by determination of relative loss of tritium-labelled adenosine for each probe. Microdialysate samples were collected at rest at week 0 (filled bar) and week 4 (open bars) for 20 min. Values are means ± SEM ($n = 9$).

through the described effect of NO on VEGF expression (Tsurumi *et al.* 1997). However, in this study there was a clear dissociation between the effect of training on the levels of eNOS and VEGF protein, as eNOS protein levels were increased whereas VEGF protein levels remained unaltered. We have previously observed a similarly divergent effect of training on VEGF and eNOS protein in subjects with hypertension where training was found to increase VEGF protein but not eNOS protein (Hansen *et al.* 2010, 2011). Thus, although shear stress is known to induce both increased VEGF and eNOS protein expressions there may be other factors of greater importance for their regulation in response to training.

To determine the functional effect of the muscle interstitial fluid we added interstitial fluid, obtained at rest and during acute exercise, to endothelial cells in culture and examined their rate of proliferation. The rate of endothelial cell proliferation was found to be higher when interstitial fluid obtained during acute exercise was added to cells. Muscle interstitial fluid obtained during acute exercise, performed after the training period, had the same cell proliferative effect as that obtained pre-training. The pattern was therefore similar to that observed for VEGF levels in the muscle interstitium that also increased similarly with acute exercise before and after training. However, it is unlikely that all of the proliferative effect was due to the presence of VEGF in the interstitial fluid. In a previous study we estimated the role of VEGF for the cell proliferative effect of the muscle interstitium by inactivating VEGF with a neutralizing antibody and found that approximately half of the effect was related to the level of VEGF in the interstitium (Hansen *et al.* 2010). Therefore, proliferative compounds in addition to VEGF appear to increase in concentration in the interstitium in response to exercise. The identity of these compounds remains to be elucidated.

Although VEGF is a key component of angiogenesis, several other pro- and anti-angiogenic compounds are also involved in the regulation. To obtain a further understanding of how the gene expression of pro- and anti-angiogenic compounds may be altered with acute exercise and training, we examined the mRNA content of a number of compounds, proposed to be of importance in angiogenesis. Destabilization and remodelling of the capillary, which occurs during capillary growth, is partly

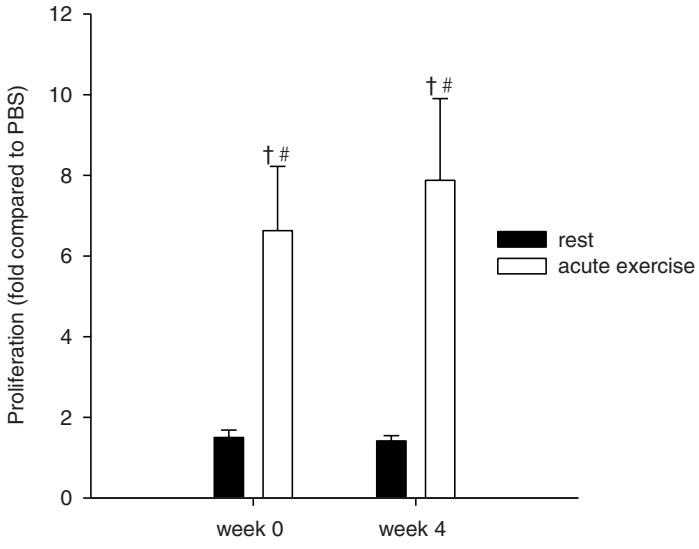

Figure 4. Effect of skeletal muscle microdialysate on proliferation of cultured endothelial cells
Proliferation of human umbilical vein endothelial cells determined by incorporation of BrdU, after addition of skeletal muscle microdialysate collected at rest and during moderate continuous exercise before and after 4 weeks of moderate continuous training. Microdialysate samples were collected at rest (filled bars) and during 0–60 min (open bars) of exercise. Values are means ± SEM ($n = 8$). †$P < 0.001$ *vs.* PBS; #$P < 0.05$ *vs.* rest. PBS = proliferation of endothelial cells with addition of the microdialysis perfusate consisting of PBS. PBS values were set to 1 and are not shown.

regulated by an increase in Ang2 with unaltered or reduced levels of Ang1. We observed a marked increase in Ang2 mRNA with no significant alteration in Ang1 mRNA, and a consequent increase in the Ang2/1 ratio in response to an acute exercise bout before training. The angiopoietin receptor Tie2 mRNA level was significantly increased with acute exercise only before training. Our data contradict previous findings in humans. Gustafsson and co-workers (2007) reported no effect of an acute exercise bout on Ang1 or 2 mRNA either under control conditions or restricted flow conditions. Gavin *et al.* (2007) showed an increase in Tie2 mRNA but no change in Ang2/Ang1 with acute resistance exercise, despite evidence of an angiogenic response. However, in rats a significant increase in the Ang2/Ang1 mRNA ratio as well as Tie2 mRNA in response to exercise has been reported (Lloyd *et al.* 2003). The present data clearly show that the transcriptional activity of the angiopoietin system is up-regulated in human muscle in response to acute exercise, and suggests that training may blunt the effect of Ang2 by reducing the exercise-induced mRNA response of Tie2.

Matrix metalloproteinases (MMPs) participate in remodelling of the extracellular space and degradation of the basement membrane surrounding the capillary. In this study we found that MMP9 mRNA amounts were higher after acute exercise indicating ongoing extracellular remodelling. However, MMP2 mRNA levels remained unaltered in accordance with previous observations in humans (Rullman *et al.* 2007; Hoier *et al.* 2010). Nevertheless, the lack of increase in MMP2 mRNA after exercise is in contrast to findings in rodents where MMP2 shows a clear up-regulation with muscle activity (Rivilis *et al.* 2002; Williams *et al.* 2006*b*). These findings suggest that MMP2 may be more stable in humans than in

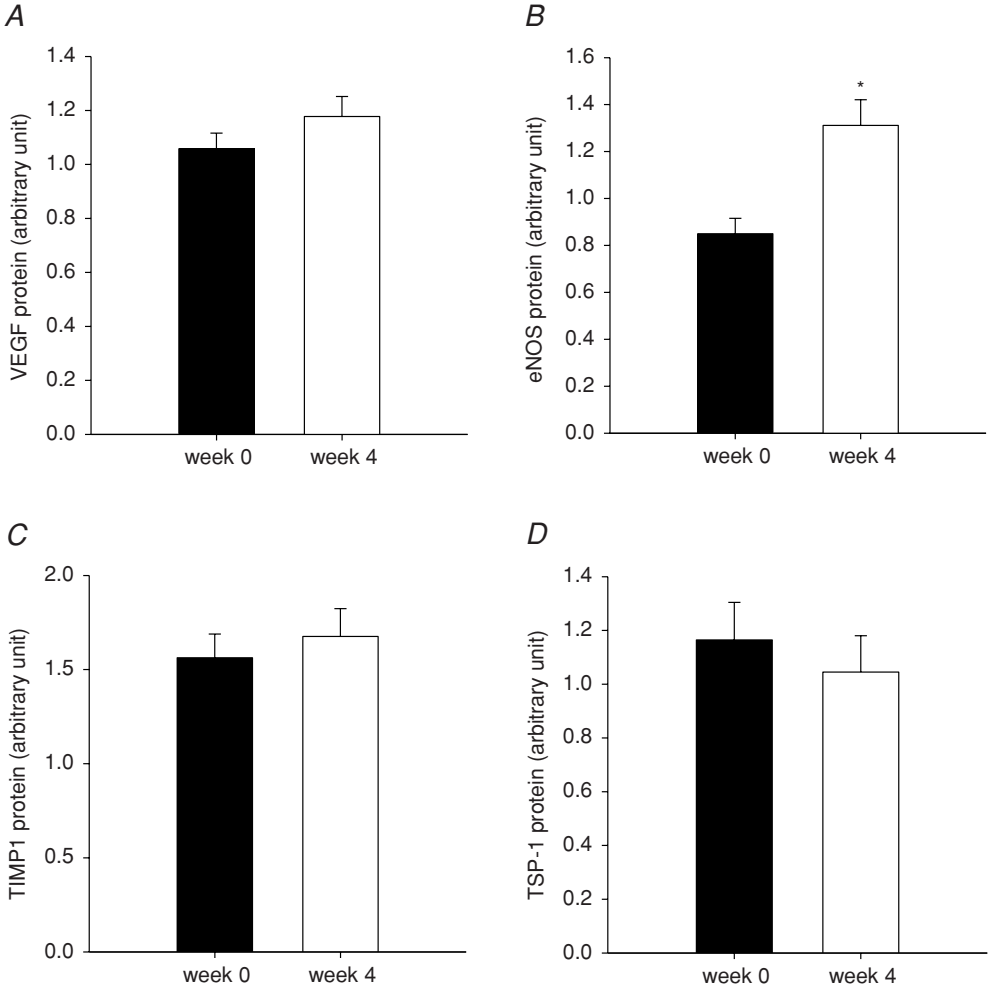

Figure 5. Resting levels of VEGF, eNOS, TIMP1, and TSP-1 protein in skeletal muscle tissue before and after 4 weeks of moderate exercise training
Resting levels of VEGF (*A*), eNOS (*B*), TIMP1 (*C*), and TSP-1 (*D*) protein before and after 4 weeks of moderate continuous training. The protein levels were determined by Western blot and results are expressed as net intensity normalized to human standards (arbitrary units). Values are means ± SEM ($n = 14$). *$P < 0.05$ *vs.* week 0.

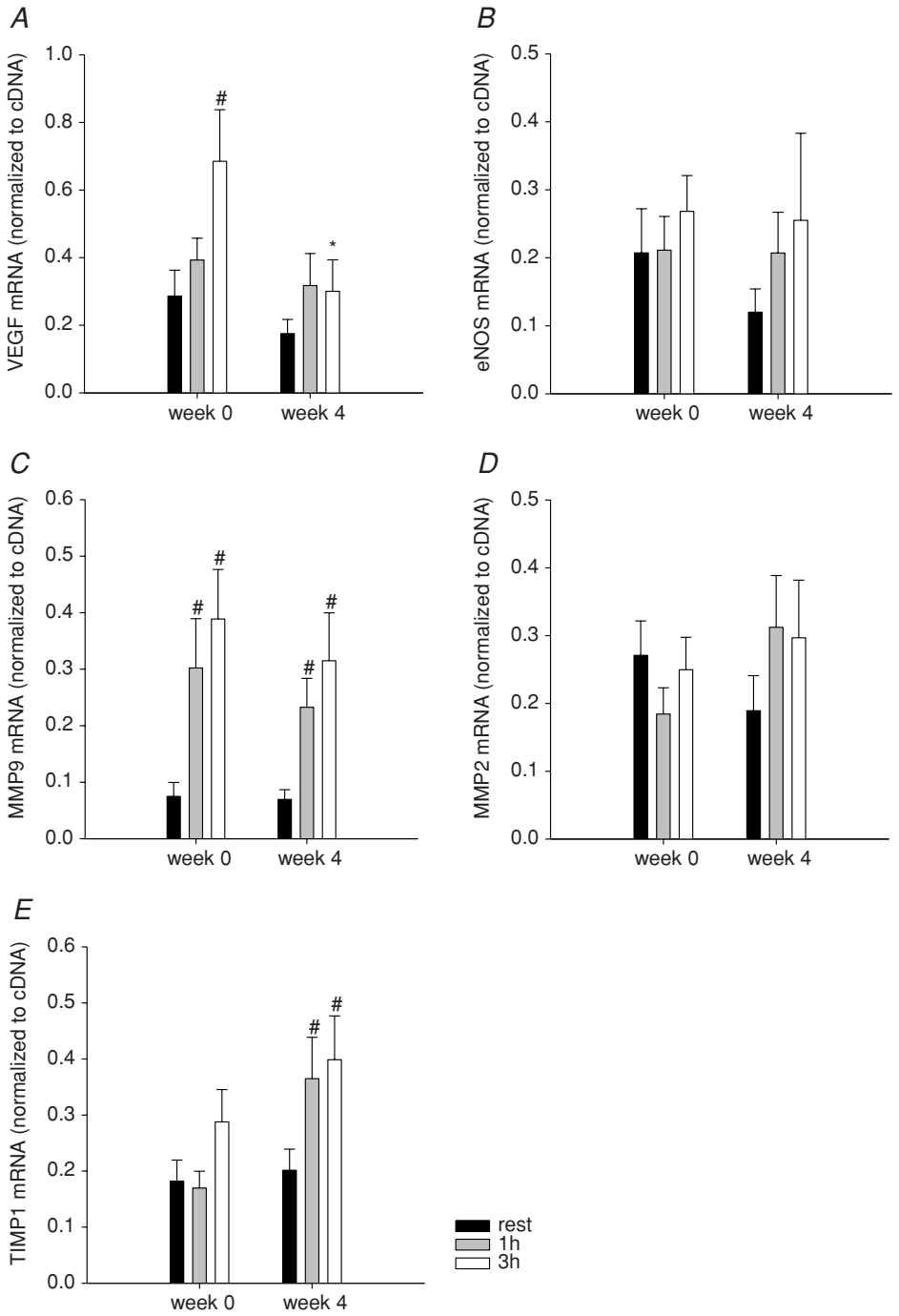

Figure 6 (continued overleaf). Content of VEGF, eNOS, MMP9, MMP2, TIMP1, Ang2, Ang1, Ang2/Ang1 ratio, Tie2, TSP-1 and PF4 mRNA in human skeletal muscle tissue before and after 4 weeks of training
The mRNA content of VEGF (*A*), eNOS (*B*), MMP9 (*C*), MMP2 (*D*), TIMP1 (*E*), Ang2 (*F*), Ang1 (*G*), Ang2/Ang1 ratio (*H*), Tie2 (*I*), TSP-1 (*J*) and PF4 (*K*) was determined in skeletal muscle tissue during moderate continuous exercise before and after 4 weeks of moderate continuous training. Muscle biopsies were obtained from m. vastus lateralis at rest (black bars), and 1 h (grey bars) and 3 h (open bars) after exercise. mRNA levels were determined with real-time RT-PCR, and data are presented relative to cDNA. Values are means ± SEM ($n = 14$). #$P < 0.05$ *vs.* rest; *$P < 0.05$ *vs.* 3 h week 0.

rodents. An interesting observation in our study was that mRNA levels of the MMP inhibitor TIMP1 was found to be elevated in response to acute exercise after, but not before, training. This finding indicates that the modulation/inhibition of extracellular remodelling after the training period was greater when substantial capillary growth had already occurred.

The level of TSP-1 mRNA increased with acute exercise to a similar extent before and after training. This finding is in line with a previous finding in rats showing an increase in TSP-1 mRNA levels after exercise, with a similar increase occurring before and after training (Olfert *et al.* 2006). TSP-1 is an anti-angiogenic factor that has been shown to be important for the regulation of capillarization in

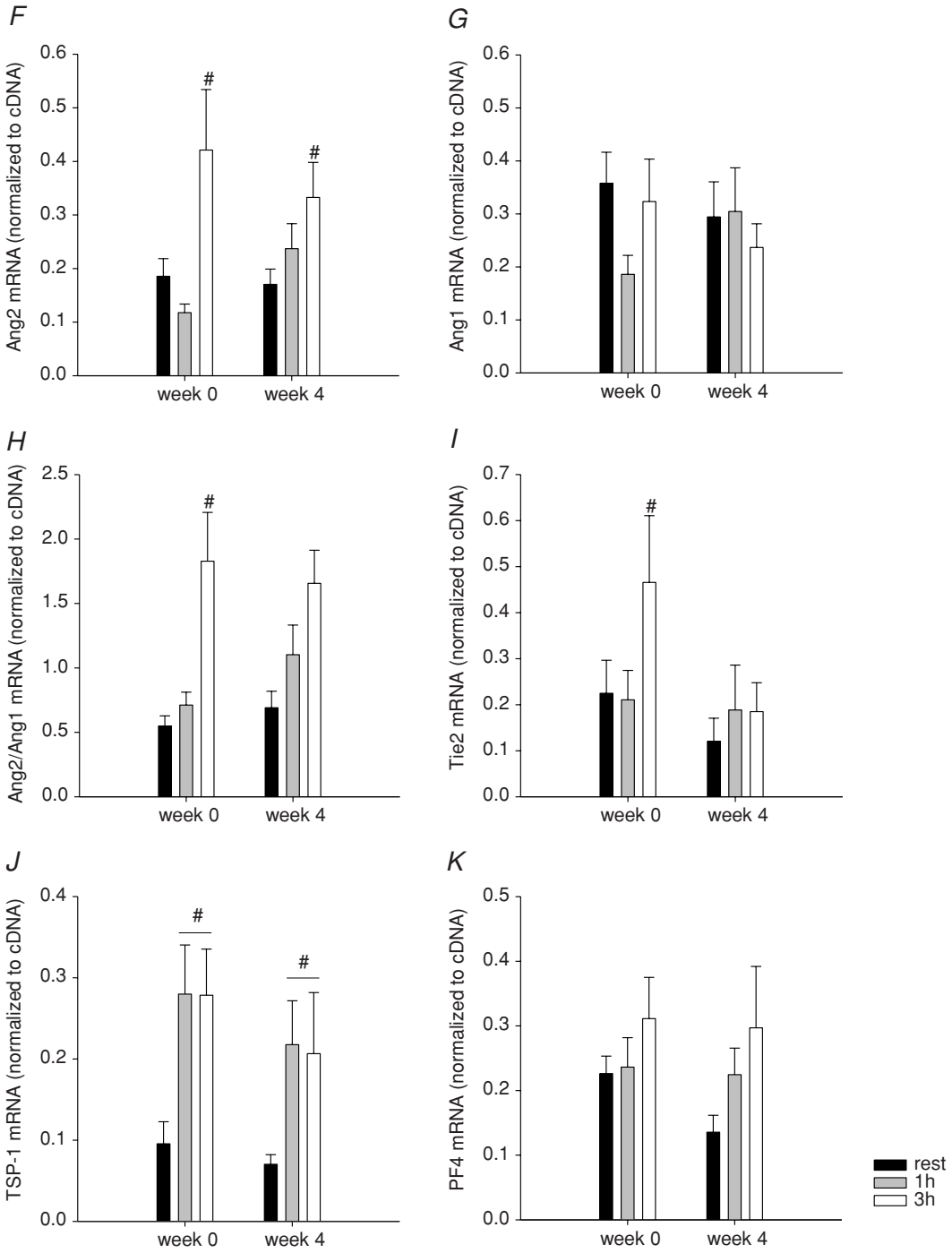

Figure 6 continued.

skeletal muscle (Malek & Olfert, 2009) in part by inhibiting MMPs (Lawler, 2000). Thus, TSP-1 has a similar role to TIMP1 in modulating the activity of MMPs and thereby capillary growth. However, whereas TIMP1 mRNA only increased with acute exercise after training, TSP-1 mRNA was increased to a similar extent before and after training. The reason for this discrepancy is unclear but may suggest different regulatory mechanisms or somewhat divergent roles for these two inhibiting factors.

In contrast to our hypothesis, the protein amounts of VEGF, TSP-1 and TIMP1 were similar before and after training, despite the increase in mRNA levels in response to acute exercise. This finding suggests that pro- and anti-angiogenic factors primarily respond acutely and transiently to exercise and that a more long-term change in basal protein levels is not necessarily desired for well-controlled regulation of angiogenesis.

In conclusion, the present study shows that acute exercise leads to an increased gene expression of both pro- and anti-angiogenic factors, and we propose that a transient up-regulation of anti-angiogenic factors serves to modulate the angiogenic process to prevent excess capillary growth. Moreover, after a 4 week period of training leading to increased capillarization, the basal protein and mRNA levels, as well as the acute exercise-induced increase in mRNA for several angiogenic factors, remained largely unaltered, suggesting a similar level of angiogenic potential in untrained and trained muscle.

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Author contributions

The experiments were carried out at Copenhagen Muscle Research Centre, Department of Exercise and Sport Sciences, University of Copenhagen, Denmark. B.H., N.N., S.A., J.B. and Y.H. contributed to the conception and design of the experiments, B.H., N.N., S.A., L.J., L.N., J.B. and Y.H. to the collection, analysis and interpretation of data, and B.H., N.N., S.A., L.J., L.N., J.B. and Y.H. to drafting the article or revising it critically for important intellectual content. All authors approved the final version of the manuscript.

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