Passive leg movement enhances interstitial VEGF protein, endothelial cell proliferation, and eNOS mRNA content in human skeletal muscle

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Hellsten Y, Rufenner N, Nielsen JJ, Høier B, Krstrup P, Bangsbo J. Passive leg movement enhances interstitial VEGF protein, endothelial cell proliferation, and eNOS mRNA content in human skeletal muscle. Am J Physiol Regul Integr Comp Physiol 294: R975–R982, 2008. First published December 19, 2007; doi:10.1152/ajpregu.00677.2007.—The present study used passive limb movement as an experimental model to study the effect of increased blood flow and passive stretch, without enhanced metabolic demand, in young healthy male subjects. The model used was 90 min of passive movement of the leg leading to a 2.8-fold increase (P < 0.05) in blood flow without a significant enhancement in oxygen uptake. Muscle interstitial fluid was sampled with microdialysis technique and analyzed for vascular endothelial growth factor (VEGF) protein and for the effect on endothelial cell proliferation. Biopsies obtained from the musculus vastus lateralis were analyzed for mRNA content of VEGF, endothelial nitric oxide synthase (eNOS), and matrix metalloproteinase-2 (MMP-2). The passive leg movement caused an increase (P < 0.05) in interstitial VEGF protein concentration above rest (73 ± 21 vs. 344 ± 83 pg/ml). Addition of muscle dialysate to cultured endothelial cells revealed that dialysate obtained during leg movement induced a 3.2-fold higher proliferation rate (P < 0.05) than dialysate obtained at rest. Passive movement also enhanced (P < 0.05) the eNOS mRNA level fourfold above resting levels. VEGF mRNA and MMP-2 mRNA levels were unaffected. The results show that a session of passive leg movement, elevating blood flow and causing passive stretch, augments the interstitial concentrations of VEGF, the proliferative effect of interstitial fluid, and eNOS mRNA content in muscle tissue. We propose that enhanced blood flow and passive stretch are positive physiological stimulators of factors associated with capillary growth in human muscle.

microdialysis; angiogenesis; matrix metalloproteinase-2; vascular endothelial growth factor; endothelial nitric oxide synthase

EXERCISE TRAINING is known to increase the level of capillarization in skeletal muscle (2), thereby improving the capacity for diffusion of oxygen and nutrients. Studies on the regulation of capillarization in response to exercise and training have shown that acute exercise enhances the protein and mRNA levels of several angiogenic factors such as vascular endothelial growth factor (VEGF) (21, 31), matrix metalloproteinase-2 (MMP-2) (6, 15), and endothelial nitric oxide synthase (eNOS) (11, 34). In addition, acute exercise has been shown to cause an increase in the muscle interstitial concentration of VEGF (18, 21), which, at least in part, appears to reflect a release of VEGF from skeletal muscle cells as demonstrated in cultured muscle cells (22). During exercise, at least three physiological factors are believed to induce angiogenesis in skeletal muscle: 1) increased blood flow, and thereby shear stress; 2) mechanical stretch of the tissue; and 3) enhanced metabolism (5). In rat and mice models it has been shown that an enhanced level of shear stress, induced by the α-adrenergic receptor blocker prazosin, leads to an upregulation of VEGF mRNA and an enhanced capillarization but has no effect on MMP-2 expression in skeletal muscle tissue (25). This effect of shear stress on VEGF Furthermore appears to be dependent on nitric oxide, because prazosin treatment does not upregulate VEGF mRNA in eNOS knockout mice (4, 7). Moreover, a period of mechanical stretch/overload of the rat extensor digitorum longus muscle, achieved by unilateral extirpation of the tibialis anterior, has been shown to induce an up regulation of basal levels of VEGF and MMP-2 and an increase in capillarization (32). The effect of the metabolic signaling on angiogenesis in skeletal muscle is more difficult to separate from the other two factors, but studies on isolated tissue suggest that metabolites and compounds such as adenosine and prostanoids released by the muscle during increased metabolic demand (10, 16) enhance VEGF expression and endothelial cell proliferation (1, 26).

Passive movement of the lower leg leads to an enhancement in blood flow with a negligible effect on muscle metabolism (23). The increase in blood flow that occurs during passive movement of the leg is therefore likely to be coupled to mechanical factors, where compression and stretch of the vasculature may be primary factors causing an increase in flow and thereby shear stress.

Passive limb movement also leads to stretch of the muscle fibers in the quadriceps muscle, which could be a contributing factor in angiogenesis. It should be noted, however, that a difference between the present model and the above-described rodent model, used to study the role of mechanical stretch in angiogenesis (9), is that the present model is not paralleled by mechanical overload, and therefore the impact of stretch is likely to be smaller.

No previous human experimental model has separated the effect of increased muscle metabolism from an increase in blood flow and passive stretch to study the role of these physiological stimuli for the promotion of angiogenic factors. In the present study we utilized passive movement of the lower leg in humans as experimental model to induce an elevated level of blood flow and passive stretch, without a concurrent increase in muscle metabolism. To assess the effect of this model on selected angiogenic factors, muscle microdialysis samples were obtained for the assessment of interstitial VEGF protein concentration and the effect of interstitial fluid on growth factors.
endothelial cell proliferation, and muscle biopsy samples were obtained for the determination mRNA levels of VEGF, eNOS, and MMP-2.

MATERIALS AND METHODS

Subjects

Seven healthy male subjects with a mean age of 24.7 (range: 19–31) yr and mean weight of 81.1 (67–98) kg participated in the study (experimental group). In another group of 11 healthy male subjects [age: 23.8 (21–26) yr, weight: 76.9 (67.9–87.3) kg], blood flow and oxygen uptake were measured during passive movement of the leg (blood flow group). The subjects were nonsmokers and habitually active but performed no regular training.

Microdialysis Probes

The semipermeable fibers used to construct the microdialysis probes had a molecular mass cutoff of 960 kDa and inner and outer diameters of 0.34 and 0.44 mm, respectively (Asahi Medical, Tokyo, Japan). The probes were made by gluing each end of a fiber 2 cm into a hollow nylon tube (0.50-mm inner diameter and 0.63-mm outer diameter; Portex SIMS, Hythe, UK). A 6-0 suture (Vicryl; Ethicon, Norderstedt, Denmark) was advanced inside and glued to the ends of the probe to provide tensile strength. The distance between the two nylon tubes was 4 cm, exposing 4 cm of the fiber.

Experimental Design

On at least one occasion before the experimental day, all included subjects were acquainted with the passive knee extensor movement. For 48 h before the experiment, the subjects were requested not to ingest caffeine-containing drinks. For 48 h before the experiment, the subjects were requested not to ingest caffeine-containing drinks.

Experimental group. On the morning of the experiment, the subjects of the experimental group had a light breakfast. The subjects rested in the supine position, and after local anesthesia was administered, a biopsy was obtained from the musculus vastus lateralis of the experimental leg. Before the insertion of the microdialysis probes into the musculus vastus lateralis, the skin, subcutaneous tissue, and fascia close to both the insertion and exit points were anesthetized with lidocaine (Xylocaine; 20 mg/ml). With the use of a 17-gauge/45-mm Venflon intravenous catheter, three microdialysis probes were inserted. The direction of the microdialysis probes was aligned with the direction of the muscle fiber. The microdialysis probes were perfused via phosphate-buffered saline, pH 7.4, with a high-precision syringe pump (CMA 102; Carnegie Medicine, Solna, Sweden) at a rate of 5 μl/min. A small amount (2.7 nM) of 2-3H-labeled adenosine (2-3H)Ado was included in the perfusate for the calculation of probe recovery. The main purpose of this determination of probe recovery was to account for differences in recovery from rest to passive movement. Suction was connected to the outflow tube of the 960-kDa probes to prevent fluid loss from the probe during the experiment (18).

Approximately 20 min after the placement of the probes, the subjects performed 10 min of light exercise at 20 W, and they then rested in the supine position for at least 45 min following the exercise. The OR of leg heart rate followed by the 45-min period of rest has been found to bring back a transient increase in ions to stable levels after the placement of probes (27). Collection of perfusate at rest was begun ~90 min after insertion of the probes and ended after 30 min. With the subject seated in an upright position, the knee was passively extended from an angle of 90 to 35° and then back to 90° again, at a rate of 60 cycles/min for 90 min. Dialysate was collected every 30 min throughout this period and during 30 min of recovery after the end of the movement. Muscle biopsies were obtained at 1, 3, and 5 h after the end of the session. All samples were immediately frozen and stored at −80°C until the time of analysis.

After collection of samples, the microdialysis was weighed, and the actual flow rate was calculated to estimate any loss of fluid or abnormal decrease in perfusion rate (PR). The relative loss for each probe was determined according to the internal reference method (19, 33) for [2-3H]Ado. The molecular PR was calculated as PR = [(dpminfusate − dpmanalyte)/dpminfusate], where dpm denotes disintegrations per minute. The 3H activity (in dpm) was measured on a liquid scintillation counter (Tri-Carb 2000; Copenhagen, Denmark) after addition of the infusate and dialysate (5 μl each) to 3.0 ml of Ultima Gold scintillation liquid (Packard Instruments, Groningen, The Netherlands).

Blood flow group. For determination of blood flow and oxygen uptake during passive exercise, the subjects of the blood flow group rested in the supine position, and two catheters were placed under local anesthesia. One catheter, for the collection of arterial blood samples, was placed antegrade into the femoral artery of the nonexercising (left) leg with the tip positioned ~2 cm proximal to the inguinal ligament. The other catheter, for collection of venous blood samples and measurement of blood temperature, was placed antegrade in the femoral vein of the experimental leg (right) with the tip positioned 2 cm distal to the inguinal ligament proximal to the saphenous vein. A thermistor (Edslab, T.D. Probe, 94-030-2.5F; Baxter, Allerod, Denmark) for measurement of blood temperature was advanced ~8 cm beyond the tip of the venous catheter for measurement of venous blood temperature for the calculation of thigh blood flow. The catheter was perforated with four side holes to facilitate perfusate dispersion. Blood flow was assessed with the thermodilution technique as previously described by Andersen and Saltin (3). Measurements of flow, blood oxygen content, and blood lactate were determined at rest, during passive movement of the leg, and during knee extensor exercise at 25 W. The frequency was 60 times per minute for both the passive and the active exercise. Infusion rates of ice-cold saline were 30 ml/min at rest and 120 ml/min during passive and active exercise.

Electromyographic Activity

Electromyographic (EMG) activity was determined on four subjects during maximal voluntary contraction (MVC) and during 90 min of passive movement of the leg. Electrodes (Neuroline; 720-01-J) were placed 1 cm apart on the belly of musculus vastus lateralis after palpation, shaving, and cleaning with ethanol. The signal was amplified (Astro-Med IP511 AC amplifier; Grass, West Warwick, RI) using a 30-Hz and 3-kHz high-pass filter, respectively, and the analog signal was recorded using an analog-to-digital converter system at 1 kHz (PowerLab 16/30; AD Instruments, Bella Vista, Australia). EMG signals were analyzed after applying a digital high- and low-pass filter of 10 and 500 Hz, respectively, followed by determination of the integrated EMG (iEMG) via root mean square calculation of 1-s segments (ChartPro; AD Instruments).

Before passive movement, the subjects performed three maximal voluntary isometric contractions separated by more than 10 s. All subsequent iEMGs were normalized to the highest obtained iEMG of the three MVCs. Since no visible EMG signals were apparent during passive exercise, iEMG was determined for 1-s blocks after 5, 15, 60, and 89 min of exercise. Thus the chosen time period would include at least one leg extension per flexion cycle. In case of movement artifacts not removed by the filtering procedure, periods of at least 1 s were selected less than 60 s away from the intended time point.

Dialysate VEGF Protein Measurements

Dialysate obtained from the microdialysis probes was analyzed for VEGF protein by using a Quantikine enzyme-linked immunosorbent assay kit (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s protocol. The concentration of VEGF was measured in the collected dialysate, and the concentration in the interstitium was estimated by determination of relative loss of tritium-labeled adenosine for each probe.
Dialysate Potassium Measurements

The microdialysis samples were analyzed for potassium using a flame photometer (FLM3; Radiometer, Copenhagen, Denmark) with lithium as internal standard (28).

Measurement of Endothelial Cell Proliferation

Human umbilical vein endothelial cells, supplemented in medium 200 with low serum growth supplement containing fetal bovine serum, fibroblast growth factor, heparin, and epidermal growth factor, intended for use in the culture of endothelial cells (Cascade Biologics, Portland, OR), were grown on 96-well plates for 24 h before the medium was replaced with 50 μl of microdialysate collected during the experiment, perfusate, or supplemented medium 200. The dialysate was diluted 1:1 with perfusate. None of the values exceeded the positive control. The positive control used was achieved by addition of medium 200 with growth supplement. After an additional 24 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 the manufacturer’s recommendations.

Analysis of Skeletal Muscle mRNA Content: RNA Isolation, Reverse Transcription, and PCR

Total RNA was isolated from the muscle biopsies using Trizol reagent, following the guidelines of the manufacturer (Invitrogen, Carlsbad, CA), and the final pellets were resuspended in 50 μl of diethyl pyrocarbonate-treated H2O containing 16 units of RNase inhibitor (Roche). The purity of the samples was assessed from the ratio of absorbance at 260 and 280 nm, which was always >1.9, and total RNA was quantified from the absorbance at 260 nm.

With the SuperScript II reverse transcriptase enzyme (Invitrogen), 3 μg of total RNA were reverse transcribed as previously described (29). First-strand cDNAs were diluted in nuclease-free water. The mRNA contents of eNOS, VEGF, MMP-2, and GAPDH were determined using real-time PCR (ABI Prism 7900 sequence detection system; Applied Biosystems, Foster City, CA). GAPDH and MMP-2 cDNAs were amplified using TaqMan gene expression assays (Applied Biosystems). Forward and reverse primers and TaqMan probes were designed for each of the three other genes by using human-specific sequence databases (Entrez-NIH and Ensembl, Sanger Institute), computer software (Primer Express; Applied Biosystems) and were synthesized by TAG Copenhagen (Copenhagen, Denmark). Sequences for eNOS (17) and VEGF (21) have been published previously. For each of the genes, a BLAST search revealed that sequence homology was obtained only for the target gene. TaqMan probes for eNOS and VEGF were 5’-(6)-carboxyfluorescein (FAM) and 3’-6-carboxy-4-N,N,N’N’-tetramethylrhodamine (TAMRA) labeled, whereas MMP-2 and GAPDH had a nonfluorescent quencher at the 3’ end of the probe. Prior optimization was performed as previously described (30). PCR amplification was performed (in triplicate) in a total reaction volume of 10 μl. An identical PCR cycle profile was used for all genes: 50°C for 2 min, 95°C for 10 min, and 95°C for 15 s + 60°C for 1 min, for 40 cycles. The threshold cycle (Ct), reflecting the initial target mRNA content in the sample, was converted to a relative amount by using a standard curve obtained by running a serial dilution of pooled cDNA samples. For each sample, the amount of target gene mRNA was normalized to the GAPDH mRNA content. The effect of the experimental conditions on the level of GAPDH mRNA was statistically determined, and no significant effect was found of the antioxidant compared with placebo treatment or of exercise on the expression of GAPDH mRNA.

Blood Analysis

All blood samples were collected in 2-ml syringes containing heparin and were immediately placed in ice slurry until analyzed.

Oxygen saturation and hemoglobin level were determined with an ABL 510 (Radiometer). Lactate was determined in triton-treated samples with YSL 2300 (Yellow Springs Instruments, Yellow Spring, OH).

Statistics

The muscle content of mRNA was calculated as arbitrary mRNA divided by the amount of GAPDH mRNA. The mRNA data as well as the VEGF protein and the proliferation data were analyzed with Friedman’s one-way repeated-measures ANOVA on ranks with time as variable. Significant main effects were determined with a Student-Newman-Keuls post hoc test. Blood flow and oxygen uptake at rest and during passive movement were analyzed with Student’s t-test. Results are means ± SE if not otherwise stated. The level of significance was set to P < 0.05.

RESULTS

Interstitial VEGF Protein

The concentration of VEGF was higher in the dialysate collected at rest (73 ± 21 pg/ml) compared with the perfusate (0 pg/ml) (P < 0.05) and it was further elevated to a peak level of 344 ± 83 pg/ml (P < 0.01) during passive movement of the leg (Fig. 1). The VEGF protein concentration in recovery after the movement was higher than at rest (P < 0.05) and was not different from the concentration during passive movement. The estimated recovery of the probes using radioactively labeled adenosine was 0.40 ± 0.02 at rest and 0.55 ± 0.04 during passive movement.

Dialysate Potassium

Potassium was analyzed in all microdialysis probe collections to assess whether any of the probes displayed abnormally high potassium values indicating tissue damage. Potassium values of the probes were all within normal variation, ranging from 4 to 8 mM. Linear regression of VEGF and potassium...
interstitial concentrations showed, moreover, no relationship between these two compounds (\( r^2 = 0.189 \)).

**Effect of Interstitial Fluid on Proliferation of Endothelial Cells**

Analysis of the effect of the interstitial fluid on proliferation of endothelial cells in culture showed that dialysate obtained at rest had a similar proliferative effect on endothelial cells as perfusate fluid (Fig. 2). Dialysate obtained during passive movement of the leg enhanced the proliferation of endothelial cells by 3.2-fold (\( P < 0.01 \); Fig. 2). The proliferative effect of dialysate obtained during recovery was lower (\( P < 0.05 \)) than that of dialysate obtained during passive exercise.

**Muscle mRNA Content of eNOS, VEGF, and MMP-2**

The content of eNOS mRNA was higher (\( P < 0.05 \)) at 1, 3, and 5 h after passive movement of the leg than at rest, with a 4.0-fold increase (\( P < 0.01 \)) occurring at 3 h (Fig. 3A). The VEGF and MMP-2 mRNA contents were similar before and after passive movement (Fig. 3, B and C).

**Blood Flow, Oxygen Uptake, and Blood Lactate Exchange**

Blood flow at rest was 469 ± 54 ml/min and increased 2.8-fold during passive movement of the leg to 1,298 ± 97 ml/min (Fig. 4A). The arteriovenous O2 difference was lower (\( P < 0.05 \)) during passive exercise than at rest (35 ± 7 vs. 55 ± 11 ml/l; Fig. 4B). The oxygen uptake at rest was 27 ± 8 ml/min, which was not different from the oxygen uptake during passive movement (46 ± 9 ml/min; Fig. 4C). The corresponding values during knee extensor exercise at a moderate intensity (25 W) were as follows: leg blood flow, 3,192 ± 263 ml/min; oxygen extraction, 126 ± 5 ml/l; and oxygen uptake, 392 ± 30 ml/min. There was no significant exchange of blood lactate at rest (0.02 ± 0.02 mmol/min) or during passive movement (0.02 ± 0.01 mmol/min). For comparison, during the 25 W of exercise, there was a significant release of lactate from the muscle of 2.35 ± 0.21 mmol/min (\( P < 0.05 \)).

![Fig. 2](image-url)  
Fig. 2. Effect of muscle interstitial fluid on the proliferation of endothelial cells in culture. Microdialysis probes (960-kDa cutoff) were placed in the musculus vastus lateralis of human subjects and perfused at a flow rate of 5 µl/min with a PBS buffer (pH 7.4). Microdialysate samples were collected every 30 min at rest and during and after passive movement of the leg. The dialysate samples were added to human umbilical vein endothelial cells (HUVFCS) in culture. For the determination of proliferation, bromodeoxyuridine (BrdU) was added to the cultures and incorporation of BrdU into the DNA was detected using an immunoassay. Values are means ± SE (n = 7). *P < 0.05 vs. control.

![Fig. 3](image-url)  
Fig. 3. Content of endothelial nitric oxide synthase (eNOS), VEGF, and matrix metalloproteinase-2 (MMP-2) mRNA in human skeletal muscle before and after a session of passive leg movement. The contents of eNOS (A), VEGF (B), and MMP-2 mRNA (C) were determined in skeletal muscle tissue before and at 1, 3, and 5 h after a 90-min session of passive knee extension movement of the lower leg. Muscle biopsies were obtained from musculus vastus lateralis. mRNA levels were determined with real-time RT-PCR, and data are presented in relation to GAPDH mRNA. Values are means ± SE (n = 7). *P < 0.05 vs. control.
EMG Activity

Measurements of EMG in musculus vastus lateralis during the 90 min of passive movement of the leg showed negligible activity (Fig. 5).

DISCUSSION

The present study used passive movement of the lower leg as a novel experimental human model to study the response of angiogenic factors to enhanced blood flow and passive muscle stretch, without a concomitant increase in muscle metabolism. The model was found to promote VEGF release, enhance the effect of the interstitial fluid on endothelial cell proliferation, and elevate eNOS mRNA content in skeletal muscle. Thus this study demonstrates that enhanced blood flow and/or passive stretch is/are likely to be important physiological signals for the promotion of factors related to capillary growth in human muscle.

Exercise involves three different physiological stimuli that can induce capillary growth processes in skeletal muscle, two of which are of a mechanical nature, shear stress induced by blood flow and longitudinal stretch of the tissue, and a third of metabolic nature and related to the increased energy expenditure during exercise (5). By utilizing an experimental model in humans in which primarily blood flow and passive stretch, but not contractile activity and metabolism, were significantly altered, this study has demonstrated that elevation in blood flow and/or passive stretch is/are likely to be important stimulatory signals for these factors related to angiogenesis in human muscle. This finding is in accordance with previous observations on animals where an enhanced level of angiogenic factors and increased capillarization have been observed in rats with chronically elevated blood flow achieved by treatment with an α-adrenergic blocker (8, 25). However, in contrast to the previous investigations that have used chronic elevation of blood flow for days as a model (8, 25, 32), the current study demonstrates that even a short-term (90 min) increase in blood flow is sufficient to stimulate factors associated with capillary growth.

The concentration of VEGF protein in the interstitium increased severalfold during the passive movement of the leg in the current study. Although a statistical comparison between the two separate studies was not performed, it is noteworthy that the order of magnitude of the VEGF increase was similar to that previously observed in the interstitium of young males.
during knee extensor exercise performed at a moderate intensity of 30 W (18, 20). This observation is of note because it suggests that it is mainly the effect of shear stress/passive stretch that leads to the increase in interstitial VEGF and that muscle activity is not essential for this effect. This does not exclude that muscle activity/metabolism can induce an enhancement in interstitial VEGF but suggests that there is no synergistic effect when flow and passive stretch are combined with the metabolic factor during exercise. Little is known with regard to the control of muscle interstitial VEGF protein. Studies on rat skeletal muscle cells in culture have shown that muscle cells release VEGF in response to contraction (22), and considering the relatively large proportion of skeletal muscle fibers in muscle tissue, it is likely that a reasonable fraction of the increase in muscle interstitial VEGF originates from release from the muscle fibers. The observation that the increase in interstitial VEGF has already begun within 30 min after the onset of exercise (18) suggests, moreover, that the increase in interstitial VEGF is not dependent on protein synthesis and points at a release from storage sites within the muscle cells or via proteolytic cleavage. The signaling mechanism for the release of VEGF from muscle cells remains to be elucidated; however, adenosine is a potential candidate, since it has been shown to induce VEGF release from skeletal muscle cells (20) as well as endothelial cells (13) and is formed extracellularly in muscle during contraction (24). It should be mentioned that a potential source of interstitial VEGF could be plasma. However, Rullman et al. (32a) have shown that during an exercise bout of an hour, there is an initial release of VEGF from the muscle, followed by no exchange as exercise continues. Thus a small release of VEGF from muscle to plasma may occur during exercise, but it does not appear likely that plasma VEGF is a source of muscle interstitial VEGF.

The dialysate fluid obtained from the muscle at rest and during passive movement of the leg was added to endothelial cells in culture to assess the effect of the interstitial fluid on endothelial cell proliferation. In accordance with our findings for VEGF, the effect of dialysate obtained during passive movement was enhanced in the same order of magnitude as dialysate obtained during exercise at 30 W in previous studies (18, 22), suggesting that the increases in blood flow/passive stretch are important for the proliferative effect of the fluid. The enhanced proliferation with dialysate from the passively moved muscle is likely to be explained in part by VEGF and in part by other interstitial compounds. Preliminary data from our laboratory on the effect of a VEGF-neutralizing antibody on the proliferative effect of microdialysate obtained during passive movement indicated that ~40% of the proliferative effect was due to VEGF. The value is an estimate, because there was only sufficient microdialysate available to make determinations on four samples. This estimate is, nevertheless, in good agreement with our observation in a previous study examining microdialysate from probes with a dialysis membrane cutoff of 5 kDa, which does not allow for entry of VEGF, with microdialysate from probes with a 960 kDa cutoff, allowing for VEGF passage (18). The latter study indicated that VEGF was responsible for ~40% of the proliferative effect of the interstitial fluid, and thus we estimate that more than one-half of the proliferative effect appears to be due to other angiogenic compounds, such as adenosine and prostacyclin, that are known to be released from exercising skeletal muscle (10, 16).

These compounds could also potentially increase in the muscle interstitium with passive movement and could contribute to the enhanced proliferative effect of the muscle dialysate. It clearly would have been of interest to also examine the effect of the passive movement on such compounds; however, the low perfusion rate required in the microdialysis technique only allows for a limited sample volume, and analysis of more compounds was not possible.

A difference between the present experimental human model and that of previous animal models examining the effect of increased blood flow and muscle stretch (32) was the effect on VEGF mRNA expression. A likely reason for the lack of increase in VEGF mRNA in the present study was the brief period of time that the muscle was subjected to shear stress compared with the previous chronic stimulation models (32). Nevertheless, since exercise has been shown to transiently enhance the VEGF mRNA expression severalfold in human muscle, with peak levels occurring within the first 3 h postexercise, there appears to be a difference between the effect of passive movement compared with exercise (14, 21). On the basis of this difference, it may be speculated that the metabolic component of active muscle contraction is important for the increase in VEGF mRNA observed after exercise. This is supported by the findings of Gustafsson et al. (14a), showing that metabolic perturbation by limiting blood flow to the muscle enhances the exercise induced increase in VEGF mRNA. Our data also suggest that the increase in interstitial VEGF protein is not a factor affecting VEGF mRNA levels. This finding is in accordance with our theory that the increase in interstitial VEGF protein does not require VEGF protein synthesis and also is supported by the observation that the increase in interstitial VEGF after passive movement and exercise is rapid (18). The stimuli for VEGF release from cells and the stimuli for enhanced VEGF mRNA levels may therefore be different.

In the present study, eNOS mRNA was substantially elevated after the passive limb movement, suggesting that eNOS mRNA levels are induced by an enhanced blood flow and passive stretch. This observation agrees well with previous studies on mice in which it has been observed that eNOS in skeletal muscle is enhanced by prazosin-induced increases in blood flow (4, 36) and by surgically induced mechanical stretch (36). eNOS is believed to be involved in both angiogenesis and arteriogenesis, and recent studies using transgenic mice lacking eNOS have shown that this enzyme is of importance for angiogenesis by longitudinal splitting in response to enhanced blood flow but not for sprouting angiogenesis induced by stretch (4, 36). Because the current study determined only the acute effect of passive movement on angiogenic factors, it is difficult to relate the observed increase in eNOS mRNA levels to specific angiogenic processes; however, the change in eNOS mRNA is clearly in line with the hypothesis that enhanced blood flow and passive stretch can promote factors of importance for angiogenesis in muscle.

The experimental model in the present study was designed to examine the response of angiogenic factors to enhanced blood flow and passive stretch, without increase in muscle metabolism. The increase in blood flow during the passive movement was 2.8-fold, which is similar to the increase in blood flow observed in rodents given prazosin. The level of passive stretch was not directly measured, but the range of movement was...
~55° where the muscle fibers of the quadriceps upon forward movement are shortened and then stretched again upon return. In contrast to the rodent model of surgical extirpation (9), this model of passive stretch does not include muscle overload and therefore probably represents a comparatively smaller stimulus. There was no significant difference in leg oxygen uptake between rest and the passive movement, and no release of lactate was observed. Negligible muscle activation during the passive movement was also confirmed by measurements of EMG activity of the thigh muscle. Therefore, it appears reasonable to assume that the primary physiological stimuli in the presently used experimental model were increased blood flow and passive stretch. It should, however, be pointed out that there was a tendency for an increase in oxygen uptake with the passive movement, and therefore, a contributing role of metabolism for the response in angiogenic factors cannot be excluded. It should be noted, however, that the oxygen uptake measured during the passive movement is likely to reflect the oxygen uptake of the whole thigh (6–9 kg; Ref. 12). This is in contrast to the knee extensor exercise situation, where the oxygen uptake increases primarily in the active quadriceps muscle (2–3 kg; Ref. 12).

Therefore, it may be estimated that the metabolic increase during the passive exercise was <5% of that seen during 30 W of exercise. Nevertheless, the reason for the trend in increased oxygen uptake is intriguing and may suggest that there is a small energy cost related to passive stretch of the muscle. Further studies are required to examine this possibility, and one model, apart from the present EMG measurements, that could be used to rule out the role of muscle activity would be to apply epidural block to inhibit central activation of the muscles during passive movement (35).

**Perspectives and Significance**

The present study shows that passive limb movement, leading to an increase in blood flow and passive stretch, provides physiological signals of importance for the regulation of factors associated with angiogenesis. This finding is novel and of importance for the basic understanding of the processes of capillary growth. The results also suggest that a worthwhile future direction would be to examine whether a period of repeated sessions of passive movement of a limb could influence capillary growth processes. Such an intervention could prove to be useful for individuals with long-term injuries of a limb and for patients with restricted physical capacity that suffer from capillary rarefaction in skeletal muscle.

In conclusion, passive movement of the leg induces an acute enhancement of muscle blood flow and involves passive stretch of the tissue, without a concomitant increase in muscle metabolism. Use of this experimental model results in an increase in extracellular VEGF protein and other proliferative compounds, and in an upregulation of eNOS mRNA in human muscle; factors that all are associated with angiogenic processes. This study demonstrates for the first time in humans that enhanced blood flow and passive stretch are important physiological stimuli for factors associated with capillary growth in skeletal muscle. The findings do not exclude muscle activity and enhanced metabolism as being important stimuli for angiogenic processes but suggest that an enhanced muscle metabolism during contraction is not essential for the induction of specific angiogenic factors.

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