Angiogenic response to passive movement and active exercise in individuals with peripheral arterial disease

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Hoier B, Walker M, Passos M, Walker PJ, Green A, Bangsbo J, Askew CD, Hellsten Y. Angiogenic response to passive movement and active exercise in individuals with peripheral arterial disease. J Appl Physiol 115: 1777–1787, 2013. First published October 24, 2013; doi:10.1152/japplphysiol.00979.2013.—Peripheral arterial disease (PAD) is caused by atherosclerosis and is associated with microcirculatory impairments in skeletal muscle. The present study evaluated the angiogenic response to exercise and passive movement in skeletal muscle of PAD patients compared with healthy control subjects. Twenty-one PAD patients and 17 aged control subjects were randomly assigned to either a passive movement or an active exercise study. Interstitial fluid microdialysate and tissue samples were obtained from the thigh skeletal muscle. Muscle dialysate vascular endothelial growth factor (VEGF) levels were modestly increased in response to either passive movement or active exercise in both subject groups. The basal muscle dialysate level of the angiostatic factor thrombospondin-1 protein was markedly higher (P < 0.05) in PAD patients compared with the control subjects, whereas soluble VEGF receptor-1 dialysate levels were similar in the two groups. The basal VEGF protein content in the muscle tissue samples was ~27% lower (P < 0.05) in the PAD patients compared with the control subjects. Analysis of mRNA expression for a range of angiogenic and angiostatic factors revealed a modest change with active exercise and passive movement in both groups, except for an increase (P < 0.05) in the ratio of angiopoietin-2 to angiopoietin-1 mRNA in the PAD group with both interventions. PAD patients and aged individuals showed a similar limited angiogenic response to active exercise and passive movement. The limited increase in muscle extracellular VEGF combined with an elevated basal level of thrombospondin-1 in muscle extracellular fluid of PAD patients may restrict capillary growth in these patients.

intermittent claudication; skeletal muscle; microdialysis; capillary; vascular endothelial growth factor

PERIPHERAL ARTERIAL DISEASE (PAD) is an atherosclerotic condition in which blood flow to the lower limb is markedly reduced due to stenosis or occlusion of the main conduit arteries. Depending on the severity of the blood flow impairment, symptoms range from muscle pain on walking, known as intermittent claudication, through to critical limb ischemia (CLI), which may necessitate lower limb revascularization or amputation. A number of previous studies have reported a reduced capillarization in the muscles of the lower leg of PAD patients compared with healthy control subjects (1, 46), although not all studies have observed this difference (14, 32). A few studies have examined angiogenic factors in skeletal muscle in PAD (27, 40, 49, 56); however, many aspects of capillary growth regulation in PAD, including the balance between proangiogenic and angiostatic factors and the capacity for vascular endothelial growth factor (VEGF) release, remain unknown.

Capillary growth or regression is dependent on the balance between proangiogenic factors and growth-inhibiting angiostatic factors (7, 8). One of the most important proangiogenic factors in skeletal muscle is VEGF (39, 52). VEGF is present in several cell types within muscle tissue, and the largest store of VEGF exists within the myocytes, which secrete VEGF to the extracellular fluid and are, thereby, important controllers of capillary growth (23, 52). VEGF secretion from myocytes is a process that, in cell culture, has been shown to be induced both by mechanical and chemical stimuli (21, 26, 28). In human skeletal muscle tissue, secretion of VEGF from cells is evidenced by the large increases in muscle interstitial VEGF concentrations during exercise (19, 20, 22) or passive leg movement (17, 24). There are indications in the literature that VEGF secretion in response to muscle activity may be impaired in cardiovascular disease and aging. In individuals with essential hypertension, muscle contraction does not appear to induce a significant increase in muscle dialysate VEGF (15), and, in aged subjects, the increase in muscle dialysate VEGF levels is lower compared with that of young subjects (9). Considering the importance of myocyte-specific VEGF for angiogenesis in skeletal muscle (52), this impairment in VEGF secretion in essential hypertension and aged individuals is likely to have consequences for capillary growth, which is consistent with reduced muscle capillary densities in these groups. Muscle tissue levels of VEGF protein have also been found to be lower in aged compared with young individuals (48) and in hypertensive compared with healthy individuals (15) in the thigh muscle; whereas, in the gastrocnemius muscle, levels have been reported to be similar between PAD patients and matched control subjects (27). However, the relationship between basal muscle VEGF levels and interstitial VEGF levels in response to exercise has not been examined.

The angiogenic effect of VEGF in skeletal muscle is exerted through two VEGF receptors, VEGF receptor-1 (VEGFR-1) and -2 (VEGFR-2/Flik-1), where VEGFR-2 is believed to be responsible for the main angiogenic effect (35). VEGFR-2 levels have not been examined in PAD patients; however, a
previous study investigated VEGF receptor protein-1 (VEGFR-1) content in the gastrocnemius muscle of PAD patients and reported a lower baseline muscle protein VEGFR-1 level compared with controls and unaltered levels after a period of prolonged endurance training (27).

Apart from VEGF, proangiogenic factors believed to be involved in the regulation of capillary growth also include matrix metalloproteinases (MMPs) and angiopoietin-2 (Ang-2), which destabilize the capillary basement membrane, and endothelial nitric oxide synthase (eNOS), which holds several functions, including the regulation of VEGF levels (31, 45, 50). There are also several angiostatic factors that modulate or inhibit capillary growth, such as tissue inhibitor of matrix metalloproteinase (TIMP)-1, which inhibits the effect of MMPs, and angiopoietin-1 (Ang-1), which modulates growth by competing with Ang-2. Moreover, thrombospondin-1 (TSP-1) and soluble VEGFR-1 (sVEGFR-1) are angiostatic factors that inhibit the effect of VEGF (29, 33).

Angiogenic factors are regulated both by mechanical stimuli, such as shear stress and passive stretch, but also by chemical and metabolic factors, all of which are present during muscle contraction (2, 3, 5, 7, 30, 45, 47, 54, 55). In humans, the role of shear stress and passive stretch in angiogenesis has been demonstrated using passive movement of the lower limb (knee flexion and extension). In healthy young subjects, there is about a threefold increase in blood flow during passive movement without an increase in metabolism (17, 24, 34). This passive movement model also induces a component of passive stretch, with an approximate 20% increase in sarcomere length in the thigh muscle (17). In young subjects, the passive leg movement model has been found to increase the expression of angiogenic factors (17), and passive movement training over 4 wk initiates angiogenesis (24). For PAD patients who experience pain on walking, the passive movement model may prove to be a useful tool to improve leg vascularization without the pain associated with exercise.

The hypothesis of the present study was that the skeletal muscle angiogenic potential is reduced in individuals with PAD, and that the balance between proangiogenic and angiostatic factors is off-set toward a more angiostatic condition. We furthermore hypothesized that passive movement would be as effective as active exercise in stimulating angiogenic factors.

**MATERIALS AND METHODS**

**Study Overview**

Two studies were conducted: one passive movement study (study I), and one active exercise study (study II). A total of 38 subjects were included in the two studies, where 17 of the subjects were aged healthy controls, and 21 subjects had been diagnosed with PAD. The subjects were randomly assigned to the two studies: 12 PAD and 7 healthy control subjects in study I, and 9 PAD and 10 healthy control subjects in study II (Table 1). Four of the subjects participated in both studies, with at least 14 days between the experimental days.

The PAD patients had a hemodynamically significant stenosis or occlusion at the iliac or femoral arteries that was confirmed with duplex ultrasound. Ankle-to-brachial index (ABI) was calculated for each limb as the highest ankle systolic pressure divided by the highest systolic brachial artery pressure, and all patients had an ABI < 0.9, except for one patient for whom ABI was artificially elevated due to tibial vessel calcification. The patients had stable intermittent claudication and had been diagnosed at least 1 yr before inclusion in the study. The PAD patients were also classified according to lower limb pain in response to walking, where none of the participating patients reported being able to walk more than 200 m without pain. Exclusion criteria included uncontrolled conditions, including severe hypertension and unstable angina. The group of healthy control subjects was selected according to age, body mass index, and sex and had a low average daily physical activity level. The anthropometric and clinical characteristics of the subjects are summarized in Table 1. In the active study, the mean age of the PAD patients was significantly ($P < 0.05$) higher than that of the control group, and the ABI and peak power output were significantly ($P < 0.05$) lower in the PAD patients compared with the control group. In the passive study, the ABI and peak power output were significantly ($P < 0.05$) lower in the PAD

Table 1. Demographic and clinical characteristics of PAD patients and aged healthy control subjects in active exercise and passive movement groups

<table>
<thead>
<tr>
<th></th>
<th>PAD Patients</th>
<th>Healthy Controls</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Active Exercise</td>
<td>Passive Movement</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>Age, yr</strong></td>
<td>69 ± 2*</td>
<td>69 ± 2</td>
</tr>
<tr>
<td><strong>Sex (men/women)</strong></td>
<td>8/1</td>
<td>9/3</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>28 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td><strong>ABI</strong></td>
<td>0.76 ± 0.04*</td>
<td>0.67 ± 0.05*</td>
</tr>
<tr>
<td><strong>Current smoker, %</strong></td>
<td>22</td>
<td>33</td>
</tr>
<tr>
<td><strong>Past smoker, %</strong></td>
<td>89</td>
<td>100</td>
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<tr>
<td><strong>Statin use, %</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>ACE/ARB use, %</strong></td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td><strong>β-Blocker use, %</strong></td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td><strong>Antiplatelet use, %</strong></td>
<td>67</td>
<td>92</td>
</tr>
<tr>
<td><strong>Aspirin use, %</strong></td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td><strong>Peak power output during knee extensor exercise, W</strong></td>
<td>24 ± 3*</td>
<td>14 ± 3*</td>
</tr>
<tr>
<td><strong>Subjects who participated in both active and passive studies, n</strong></td>
<td>4</td>
<td>4</td>
</tr>
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</table>

Values are means ± SE or percentages; n, no. of subjects. PAD, peripheral arterial disease; BMI, body mass index; ABI, ankle brachial index; ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker. $*P < 0.05$ vs. active exercise healthy controls.
patients than in the control subjects. For purposes of determining the influence of age on basal muscle protein levels of angiogenic factors in the PAD and aged healthy controls, muscle biopsy samples were also obtained from eight young healthy male subjects. The young subjects were nonsmokers and habitually active, but performed no regular training. The anthropometric characteristics of the three groups are summarized in Table 2. In the present study, all patients had, as described above, significant stenosis or occlusion at the iliac or femoral arteries, and angiogenesis was studied in the muscle vastus lateralis (m. v. lateralis). This muscle group was selected for study as less is known about angiogenic potential in this muscle of PAD patients compared with the gastrocnemius muscle, and as the experimental model of passive exercise used in the present study is developed for this muscle. 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, Denmark, and of Royal Brisbane and Women’s Hospital HREC, Brisbane, Queensland, Australia, and the University of the Sunshine Coast Human Research Ethics Committee, Queensland, Australia.

**Experimental Protocol (Study I and II)**

On the experimental day, all subjects in study I and study II underwent the following procedures. The subject was seated in an adjustable chair that could be reclined to a supine position. After 30 min of supine rest, the skin, subcutaneous tissue, and fascia of the thigh were anesthetized by injection with Lidocain (Xylocaine; 20 mg/ml) to prepare for muscle biopsy sampling and insertion of microdialysis probes. A resting biopsy sample was obtained from the m. v. lateralis using a Bergstrom needle with suction and was immediately frozen in liquid nitrogen. A blood sample was drawn from the medial cubital vein, and the blood sample was transferred into a lithium heparinized tube. The sample was centrifuged at 14,500 rpm for 2 min, whereafter the plasma was transferred to storage tubes. All samples were stored at −80°C until further analysis. Three microdialysis probes with a molecular mass cutoff of 960 kDa were placed in the thigh muscle, as previously described (19), and perfused at a rate of 5 μl/min with phosphate-buffered saline, pH 7.4. Approximately 20 min after probe insertion, the subject performed 10 min of exercise at a power output of 10 W to reduce local edema and remove tissue debris after placement of the probes (36). After 70 min of rest, dialysate was collected for 30 min while the subject was resting. The subject then performed passive movement (study I) or active exercise (study II), respectively. Dialysate was continuously collected throughout the experiment, and the collection tubes were replaced every 30 min. All dialysate samples were immediately frozen and stored at −80°C until time of analysis. Flow rate of the microdialysis probes was calculated to estimate any loss of fluid or abnormal decrease in perfusion rate (19). Only probes with flow rates >4.0 μl/min and <6.0 μl/min were used for further analysis. At 0 and 2 h after the end of either active exercise or passive movement, a muscle biopsy sample was obtained from the thigh muscle.

**Passive movement protocol.** The subjects were seated in a knee extension ergometer and were subjected to 60 min of passive movement, extension and flexion, of the lower leg with an angle displacement of 55°, as previously described (17). The movement rate was set at 60 extensions/min. The subjects were instructed to completely relax their leg muscles during the movement. Lack of EMG activity and negligible changes in muscle oxygen uptake during passive leg movement have previously been verified in our laboratory (17).

**Active exercise protocol.** The subjects conducted eight 3-min bouts of single-leg knee extension exercise at 10 W, separated by 1 min of rest. Subjects maintained a contraction rate of 60 extensions/min aided by an audible metronome.

**Dialysate VEGF, sVEGFR-1, and dialysate and plasma TSP-1 protein measurements.** Dialysate VEGF, sVEGFR-1, and dialysate and plasma TSP-1 protein levels were determined by enzyme-linked immunosorberent assay kits, according to the protocol of the manufacturer (Quantikine Human VEGF, human sVEGFR-1/Fit-1, and human TSP-1; R&D System, Minneapolis, MN).

**Western blot analysis.** Western blot analysis was performed as previously described (20) on biopsies (∼28 mg) from m. v. lateralis. Primary antibodies used were mouse monoclonal antibody to VEGF (A-20, Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), mouse monoclonal antibody to eNOS (610297; BD Transduction Laboratories, Albertslund, Denmark; 1:500 dilution), rabbit polyclonal antibody to TSP-1 (ab85762; Abcam, Cambridge, UK; 1:2,000 dilution), goat antibody to Flk-1 (sc-19530, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution), and mouse monoclonal antibody to GAPDH (MAb 9484; Abcam, Cambridge, UK; 1:2,0000 dilution). The protein content was expressed in arbitrary units related to mean of samples on each membrane. GAPDH was used as loading control. There was no statistical change in GAPDH levels in response to the experimental interventions.

**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). First-strand cDNA was synthesized from 1 μg total RNA by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), as previously described (42). The mRNA content of VEGF, eNOS, MMP-9, MMP-2, TIMP-1, TSP-1, Ang-2, Ang-1, and Tie-2 was determined by real-time PCR (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Foster City, CA). The cDNAs were amplified using TaqMan Gene expression assays from Applied Biosystems (Foster City, CA). The cDNAs were amplified using TaqMan Gene expression assays from Applied Biosystems (Foster City, CA). For each sample, the amount of target gene mRNA was normalized to the GAPDH mRNA content. The effect of the experimental condition on the level of GAPDH mRNA was statistically determined, and no significant effect was found with the experimental interventions.

**Statistics**

All data are expressed as means ± SE. A Mann-Whitney rank sum test was performed to compare PAD and healthy aged controls for the basal level of VEGF, TSP-1, and sVEGFR-1 protein in dialysate, and TSP-1 protein in plasma, as data were not normally distributed. A one-way ANOVA was performed to evaluate the effect of PAD and age on VEGF, VEGFR-2/F1t-1, eNOS, TSP-1, and GAPDH protein.

Table 2. Demographic and clinical characteristics of PAD patients, aged healthy control subjects, and young healthy individuals

<table>
<thead>
<tr>
<th></th>
<th>PAD Patients</th>
<th>Aged Healthy Controls</th>
<th>Young Healthy Individuals</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Age, yr</td>
<td>68 ± 1*</td>
<td>59 ± 3*</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>15/2</td>
<td>9/8</td>
<td>8/8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27 ± 1</td>
<td>27 ± 1</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>ABI</td>
<td>0.75 ± 0.03*</td>
<td>1.20 ± 0.03†</td>
<td>NA</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Past smoker, %</td>
<td>94</td>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>ACE/ARB use, %</td>
<td>59</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>β-Blocker use, %</td>
<td>59</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>Antiplplatelet use, %</td>
<td>71</td>
<td>29</td>
<td>NA</td>
</tr>
<tr>
<td>Aspirin use, %</td>
<td>6</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Peak power output during knee extensor exercise, W</td>
<td>20 ± 2*†</td>
<td>36 ± 2*</td>
<td>57 ± 1</td>
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</table>

Values are means ± SE or percentages; n, no. of subjects. NA, not applicable. *P < 0.05 vs. young healthy individuals. †P < 0.05 vs. aged healthy controls.
content in the muscle biopsies. Two-way ANOVA with repeated measures was performed to evaluate the effect of time and group on the VEGF protein concentration in dialysate and on mRNA levels in the muscle biopsies. A Student-Newman-Keuls method for multiple comparisons was used to locate differences. The Spearman rank order correlation was used to describe relationships between parameters. A level of P < 0.05 was considered statistically significant.

Due to microdialysis probe failure in one PAD patient (study II) and failure to obtain muscle biopsy samples in three subjects (2 PAD patients in study I and 1 PAD patient in study II), the number of subjects varies for the included data. In addition, two PAD patients (study II) were excluded due to adverse symptoms, not related to PAD, on the day of testing.

RESULTS

Skeletal Muscle Dialysate VEGF, TSP-1, and sVEGFR-1 Protein

The basal dialysate level of VEGF was similar between the PAD patients and the aged control group (Fig. 1A). In the PAD group, the dialysate VEGF concentration increased 2.7-fold in response to active exercise (69 ± 21 to 190 ± 78 pg/ml, P < 0.05, Fig. 2B), and in the aged control group the dialysate VEGF concentration increased 3.5-fold in response to passive movement (70 ± 10 to 247 ± 87 pg/ml, P < 0.05, Fig. 2A).

The basal dialysate level of TSP-1 in the PAD group was approximately fourfold higher (219 ± 70 ng/ml, P < 0.05) than in the aged control group (54 ± 24 ng/ml, Fig. 1B). The basal dialysate sVEGFR-1 level was not different between the PAD group and the aged control group (Fig. 1C).

Plasma TSP-1 Protein Concentration

The basal plasma level of TSP-1 was similar in the PAD patients (160 ± 62 ng/ml) and the aged control group (176 ± 58 ng/ml).

Skeletal Muscle VEGF mRNA and Protein Levels

There was no difference in basal skeletal muscle VEGF mRNA content between the two groups (Figs. 3A and 4A). The VEGF mRNA level was increased 2 h after active exercise in the PAD group only, whereas passive movement did not alter the VEGF mRNA levels in either group (Figs. 3A and 4A). Basal skeletal muscle VEGF protein levels were ~27% lower (P < 0.05, Fig. 5A) in the PAD patients than in the aged control group. Compared with the healthy young control group, the PAD patients had a ~42% lower (P < 0.05) muscle VEGF...
Fig. 3. mRNA content of pro-angiogenic and angiostatic factors in the thigh muscle before and after a bout of passive movement. The mRNA content of VEGF (A), eNOS (B), MMP-9 (C), MMP-2 (D), TIMP-1 (E), TSP-1 (F), Ang-2 (G), Ang-1 (H), Ang-2-to-Ang-1 ratio (I), and Tie-2 (J) was determined in skeletal muscle tissue at rest and 0 and 2 h after 60 min of passive lower leg movement (post-pass). The muscle samples were obtained in the m. v. lateralis of control subjects (solid bars, n/H11005/7) and PAD patients (open bars, n/H11005/10). mRNA levels were determined with real-time RT-PCR, and data are presented relative to GAPDH. Values are means ± SE. #P < 0.05 vs. rest. €P < 0.05 vs. control. Due to failure to obtain muscle biopsy samples, two PAD patients were excluded.
Fig. 4. mRNA content of pro-angiogenic and angiostatic factors in the thigh muscle before and after a bout of active exercise. The mRNA content of VEGF (A), eNOS (B), MMP-9 (C), MMP-2 (D), TIMP-1 (E), TSP-1 (F), Ang-2 (G), Ang-1 (H), Ang-2-to-Ang-1 ratio (I), and Tie-2 (J) was determined in skeletal muscle tissue at rest and 0 and 2 h after 30 min of active knee extensor exercise (10 W) performed in 3-min intervals separated by 1 min of rest. Muscle samples were obtained in the m. v. lateralis of control subjects (solid bars; n = 9) and PAD patients (hatched bars; n = 6). mRNA levels were determined with real-time RT-PCR, and data are presented relative to GAPDH. Values are means ± SE. #P < 0.05 vs. rest. €P < 0.05 vs. control. †P < 0.05 vs. all other time points in same group. Three PAD patients were excluded due to adverse symptoms, not related to PAD, on the day of testing (2 subjects) and due to failure to obtain muscle biopsies during exercise (1 subject). One control subject was excluded due to insufficient muscle tissue sample during exercise.
Fig. 5. Baseline muscle protein content in human skeletal muscle tissue. Basal protein levels of VEGF (A), VEGFR-2/Flk-1 (B), eNOS (C), and TSP-1 (D) in m. v. lateralis of control subjects (solid bars; \( n = 17 \)), PAD patients (cross-hatched bars; \( n = 17 \)), and young healthy control subjects (gray bars; \( n = 8 \)). Muscle biopsies were obtained from m. v. lateralis at rest, and protein levels were determined by Western blot. Results are expressed as net intensity normalized to mean of loaded samples (arbitrary unit). Values are means ± SE. §\( P < 0.05 \) vs. all other groups. *\( P < 0.05 \) vs. young. Two PAD patients were excluded due to failure to obtain muscle biopsy samples. Two PAD patients were excluded due to insufficient amount of muscle biopsy tissue.

protein level, and the aged control group had a \( \sim 28\% \) lower \( (P < 0.05) \) VEGF protein level (Fig. 5A).

**Skeletal Muscle VEGFR-2/Flk-1 Protein Level**

Baseline skeletal muscle VEGFR-2/Flk-1 protein levels were similar in the PAD and the aged control group (Fig. 5B). Compared with the healthy young control group, the VEGFR-2/Flk-1 protein levels were \( \sim 55\% \) and \( \sim 37\% \) higher \( (P < 0.05) \) in the PAD and aged control group, respectively (Fig. 5B).

**Skeletal Muscle eNOS mRNA and Protein Levels**

Skeletal muscle eNOS mRNA content (Figs. 3B and 4B) was not different between the PAD patients and the aged control group at baseline, and the levels remained unaffected by active exercise and passive movement in both groups. eNOS protein levels were similar between the PAD and the aged group and not different from the young (Fig. 5C).

**Skeletal Muscle TSP-1 mRNA and Protein Levels**

Skeletal muscle TSP-1 mRNA content (Figs. 3F and 4F) was not different between the PAD patients and the aged control group at baseline, and the levels remained unaffected by active exercise and passive movement in both groups. The basal TSP-1 protein levels in the muscle samples were similar between the PAD patients, the aged control group, and the young healthy control group (Fig. 5D).

mRNA Levels of the MMP System

mRNA levels of MMP-9 (Figs. 3C and 4C) and MMP-2 (Figs. 3D and 4D) were similar in the aged control and the PAD group and remained unaltered with passive movement and active exercise. mRNA levels of the inhibitor of MMPs, TIMP-1, was increased \( (P < 0.05) \) at 2 h after passive movement in the PAD group, but remained unaltered with active exercise in the PAD group and with both conditions in the aged control group (Figs. 3E and 4E).

mRNA Levels of the Angiopoietin System

In the exercise study, basal mRNA levels of Ang-1 (Figs. 3H and 4H), Ang-2 (Fig. 4G), and the angiopoietin receptor Tie-2 (Figs. 3J and 4J) were similar in the PAD and the aged control group. In the passive study, basal mRNA level of Ang-2 was, however, higher \( (P < 0.05) \) in the PAD compared with the aged control group (Fig. 3G). Ang-1 mRNA was not significantly altered with passive movement and active exercise in either group (Figs. 3H and 4H). Ang-2 mRNA increased \( (P < 0.05) \) in response to active exercise in the PAD group (Fig. 4G), whereas Ang-2 mRNA remained unaltered in response to passive exercise (Fig. 3G). The ratio of Ang-2 to Ang-1 mRNA, which indicates capillary destabilization, was increased \( (P < 0.05) \) in the PAD group after both passive movement (Fig. 3I) and active exercise (Fig. 4I). In the aged control group, the Ang-2-to-Ang-1 mRNA ratio increased.
(P < 0.05) with passive exercise only and to a lesser extent (P < 0.05) than in the PAD group (Fig. 3I). Tie-2 mRNA remained unaltered in both groups and with both active exercise and passive movement (Figs. 3J and 4J).

**Relationship between Age, Activity Level, and Measured Parameters**

Baseline muscle VEGF protein in the PAD patient and aged control groups was not correlated with baseline interstitial VEGF protein (Fig. 6). There were no correlations between age and baseline or exercise-induced VEGF levels in muscle, or between age and baseline dialysate VEGF or dialysate TSP-1 levels. The physical activity level was not correlated to either baseline or exercise-induced VEGF levels in muscle or dialysate, or to baseline dialysate TSP-1 level.

**DISCUSSION**

The present study determined the angiogenic response to acute active exercise and passive leg movement in the thigh muscle of patients with PAD and aged control subjects. The overall finding was that the angiogenic response to active or passive movement was similar between the PAD patients and the control subjects for many of the parameters measured; however, some differences were observed between the groups: the basal level of TSP-1 protein in the muscle dialysate was markedly higher in the PAD patients than in the control subjects, and the ratio of Ang-2 to Ang-1 mRNA increased to a greater extent in the PAD patients. In addition, muscle dialysate VEGF levels were significantly increased with active exercise in the PAD patients, whereas there was an increase with passive movement in the control group. Combined, the higher basal extracellular TSP-1 levels, the limited increase in extracellular VEGF, and the modest change in mRNA expression of angiogenic factors upon stimulus in skeletal muscle of PAD patients suggest that the angiogenic potential is low in this patient group.

Skeletal muscle myocytes are central in the regulation of capillary growth in that they can initiate angiogenesis by secreting VEGF to the extracellular fluid where it can act on the capillary endothelial cells (26, 28). VEGF secretion, as evidenced by increases in interstitial VEGF levels, can occur in response to mechanical stimuli, such as contraction (19), passive movement (24), or in response to chemical stimuli (21). Previous studies on young healthy individuals have shown that the muscle interstitial concentration of VEGF increases approximately five- to eightfold with both active exercise and passive movement (17, 20, 22, 24). The present findings of a modest increase in dialysate VEGF levels with active exercise and passive movement in the PAD patients and in the aged control group are, thus, in contrast to previous findings in young individuals (17, 20, 22, 24). The impairment appears to be related both to the PAD condition, but also to age, as previously shown (9). One interesting finding in the present study was that the PAD patients showed a limited increase in dialysate VEGF with passive movement, but not in response to active exercise, whereas the aged control group showed an opposite pattern. At this time, we have no plausible explanation for this finding other than that the signal by which VEGF is released from muscle may be different in response to passive movement and active exercise, and the sensitivity to these signals may have been different in the PAD patients and the aged control group. The absolute level of VEGF protein in muscle is unlikely to be a main determinant of VEGF secretion, as there was no relationship between baseline individual muscle VEGF content and interstitial VEGF level in the present study (Fig. 6). The mechanism underlying VEGF secretion from myocytes is unclear, but we propose that an impairment in secretion is likely to be one of the causes behind the reduced angiogenic potential in PAD. It should be mentioned that muscle interstitial VEGF may originate from sources other than muscle fibers, such as plasma and pericytes (23). However, studies in humans in which exchange of VEGF has been determined over the exercising muscle indicate that plasma is not a significant contributor to the increase in interstitial VEGF (18, 47).

The lower protein content of VEGF in the thigh muscle of PAD patients compared with the aged controls in the present study is in contrast to previous reports that show similar protein content in thigh muscle of patients with PAD (27) and CLI (41) compared with control individuals. The explanation for the discrepancy in findings is unclear, but may be related to the severity of the condition and the level of ischemia. In the gastrocnemius of patients with CLI, VEGF levels have been reported to be upregulated (4, 44, 51). It is thus plausible that VEGF is upregulated when ischemia is severe (53), potentially via activation of the transcription factor hypoxia-inducible factor-1α. Hypoxia-inducible factor-1α has previously been shown to be upregulated in human muscle in CLI (51).

One of the aims of the present study was to compare the levels of proangiogenic and angiostatic factors in muscle interstitial fluid and muscle tissue of PAD patients and healthy control subjects. Apart from the lower levels of VEGF protein in muscle, a difference between the PAD patients and the control group was the higher basal level of TSP-1 in the muscle dialysate of the PAD patients. TSP-1 has been shown to inhibit angiogenesis in part by opposing the effects of VEGF (10, 25). TSP-1 mRNA levels in muscle tissue increase in response to acute exercise (20, 22,
38), most likely to modulate the extent of capillary growth. Moreover, genetic deletion of TSP-1 increases capillarization in mice (33). Thus the present finding of low-muscle interstitial VEGF levels, combined with high interstitial TSP-1 levels, would suggest poor conditions for capillary growth. Of note is that our findings on interstitial TSP-1 protein in the PAD patients were not paralleled by increased muscle tissue levels of TSP-1 protein or mRNA. Therefore, we determined plasma levels of TSP-1 to elucidate whether this was the source of the dialysate TSP-1. However, plasma TSP-1 levels were found to be similar in the PAD patients and the control group. The origin of the higher TSP-1 protein levels in the muscle dialysate of the PAD patients is, therefore, unclear.

The basal muscle dialysate concentration of another angiostatic factor, sVEGFR1 (29), which neutralizes the effect of VEGF, was found to be similar in the PAD patients and the aged controls, suggesting that sVEGFR-1 may not be a critical inhibitory factor for the angiogenic process in PAD. This proposition is also supported by our previous findings in the young that show that basal sVEGFR-1 in the muscle interstitial fluid is unaffected by exercise training (22).

The mRNA content of a number of angiogenic factors was determined in muscle tissue obtained at rest or after active exercise or passive movement. The main observations were that none of the factors were different between the two groups at baseline, and there was a limited response in mRNA levels with active exercise or passive movement in either group. The lack of effect of active exercise is in contrast to findings in young individuals in which significant increases in mRNA for several angiogenic factors have been found (11, 12, 16, 20, 22, 43, 47). The exception in the present study was the angiopoietin system, which showed a marked response to stimulation. In the PAD patients, Ang-2 levels and the ratio of Ang-2 to Ang-1 mRNA increased with both active exercise and passive movement. In the control group, the response in the angiopoietin system was somewhat less, and the ratio of Ang-2 to Ang-1 mRNA increased only after passive exercise. Ang-1 and Ang-2 compete for the same receptor, Tie-2, and the ratio of Ang-2 to Ang-1 mRNA increased only after passive exercise. Ang-1 and Ang-2 compete for the same receptor, Tie-2, and the ratio of Ang-2 to Ang-1 indicates a situation of destabilization of the basement membrane, which occurs during capillary growth by sprouting. Previous studies have shown an increase in the Ang-2-to-Ang-1 mRNA ratio and in Tie-2 mRNA in response to acute exercise (20, 22, 30), although one study has not observed this (13). In addition, MMPs that also are involved in the degradation of the extracellular matrix have consistently been found to increase with exercise in young subjects (20, 22, 47); however, in the present study, the MMP mRNA levels remained unaltered with exercise and passive movement in both aged groups.

In the present study, there was no difference in basal eNOS protein or mRNA content and no increase in eNOS mRNA with active exercise or passive movement in either the PAD or the aged control group. Our laboratory has previously shown that the basal eNOS protein content is similar in aged compared with young men, and that the level of eNOS is dependent on the level of physical activity (37). eNOS protein levels are found to be upregulated in response to both active exercise and passive movement training in young healthy subjects (20, 22, 24). The reason for the lack of increase in eNOS mRNA in response to active exercise and passive movement in the present PAD and aged control subjects is not clear. However, one possibility may be that endothelial dysfunction associated with aging and cardiovascular disease is associated with a reduced ability to increase protein expression in response to physiological stimuli. Alternatively, a strong stimulator of eNOS expression is shear stress (54), and both PAD patients and aged individuals have lower blood flows to the muscle during active and passive movement compared with young (34, 37), which may lead to a lower level of shear stress.

A limitation in the present study was that the two aged groups were not completely matched with regard to age and physical activity level. The mean age of the PAD patients was higher and the physical activity level was somewhat lower than that of the aged controls. Nevertheless, given the very similar angiogenic response between the PAD patients and the aged control group for most parameters measured, these differences were unlikely to have had a significant impact on the results. Moreover, there were no correlations between age or activity level and the basal levels or exercise-induced response in angiogenic factors, e.g., VEGF levels in muscle and dialysate, and dialysate TSP-1 levels. It should also be mentioned that the subjects were all examined at the same low absolute workload. As the PAD patients had less work capacity, the relative intensity was higher for them, and it cannot be excluded that this has influenced the results. Very large differences in exercise intensity can affect the gene expression response pattern; however, in humans the difference in response is limited (20, 22); thus the current difference in intensity probably had a limited effect.

In conclusion, the results of the present study suggest that individuals with PAD, as well as aged subjects, show a modest overall angiogenic response in response to both active exercise and passive movement. We propose that the limited overall angiogenic response, and in particular the low muscle dialysate concentration of VEGF, in parallel with the higher basal dialysate level of the angiostatic factor TSP-1, may be some of the explanation for microvascular rarefaction in PAD patients. This finding also suggests that capillary growth, in response to a period of regular passive movement or exercise training, may be slower in PAD patients than in healthy individuals. Nevertheless, it should be emphasized that exercise training clearly is effective in stimulating capillarization in this patient group (6, 27).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


