Angiogenesis in Skeletal Muscle Precede Improvements in Peak Oxygen Uptake in Peripheral Artery Disease Patients


Objective—Peripheral artery disease (PAD) is characterized by impaired blood flow to the lower extremities, causing claudication and exercise intolerance. The mechanism(s) by which exercise training improves functional capacity is not understood. This study tested the hypothesis that in PAD patients who undergo supervised exercise training, increases in capillary density (CD) in calf muscle take place before improvements in peak oxygen uptake (VO₂).

Methods and Results—Thirty-five PAD patients were randomly assigned to 12 weeks of directly supervised or home-based exercise training. Peak VO₂ testing and gastrocnemius muscle biopsies were performed at baseline and after training. CD (endothelial cells/mm²) was measured using immunofluorescence staining. After 3 weeks of directly supervised training, patients had an increase in CD (216±66 versus 284±77, P<0.01) but no increase in peak VO₂. However, after 12 weeks, peak VO₂ increased (15.3±2.8 versus 16.8±3.8, P<0.01), whereas in muscle, CD remained increased over baseline, but there were no changes in markers of oxidative capacity. Within subjects, CD was related to peak VO₂ before and after directly supervised training.

Conclusion—Changes in CD in ischemic muscle with training may modulate the response to training, and those changes precede the increase in VO₂. (Arterioscler Thromb Vasc Biol. 2011;31:2742-2748.)

Key Words: angiogenesis ■ exercise ■ peripheral arterial disease

Peripheral artery disease (PAD) is caused by atherosclerotic stenoses in the peripheral arterial tree that impair blood flow to the lower extremity. Recent statistics reported by the American Heart Association state that approximately 8 million patients in the United States are affected with PAD, with an even larger number remaining undiagnosed.1,2 Approximately one third of patients with PAD have intermittent claudication, defined as pain in 1 or both legs during exercise that is relieved with rest. Claudication causes severe exercise intolerance manifested by impaired walking ability and peak oxygen uptake (VO₂) values that are 50% lower compared with matched controls (similar to class II–III heart failure), which both diminishes quality of life and is associated with an increase in mortality.3 To date, the best noninvasive treatment to improve functional capacity and peak VO₂ in those who experience PAD remains supervised exercise training.4

The pathophysiology of intermittent claudication and the mechanism(s) by which exercise training improves functional capacity in patients with PAD remain poorly understood. Large conduit artery blood flow is certainly reduced relative to metabolic demand during exercise in PAD patients with claudication, but changes in calf blood flow that follow exercise training do not explain the increased functional capacity in PAD patients.5 However, less is known about the involvement of the microvasculature, which may have great impact on the metabolic potential of working muscle.6 A decreased capillary supply to skeletal muscle has been shown in PAD by others, although correlation with functional outcomes is not well established.7,8 The role of angiogenesis, which is the growth and proliferation of small blood vessels from existing vascular structures, in improving exercise capacity in PAD is not known. However, because exercise is a potent stimulus for angiogenic pathways and increased capillary density (CD),9–15 understanding whether exercise-induced angiogenesis occurs in patients with PAD is a key question.

Therefore, the rationale for this study was that exercise intolerance in PAD patients may be related to skeletal muscle microvascular perfusion, as measured by CD, and potentially improved by exercise training. This study tested the hypothesis that in PAD patients who undergo supervised exercise training, increases in CD in calf muscle occur and that these improvements take place before improvements in VO₂. We also proposed in this study that there is a relationship between CD and peak VO₂ in patients with PAD that to our knowledge has not been shown previously.

Received on: February 14, 2011; final version accepted on: August 9, 2011.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.111.230441
Subjects were recruited from the clinics and community at Duke University Medical Center and the University of Colorado School of Medicine. PAD subjects were selected who had symptom-limiting critical limb ischemia. After a subject was able to walk for 8 to 10 minutes at his or her rest or a 20% decrease in ABI after exercise or angiographic evidence of PAD, Subjects were required to be on a stable medical regimen including statin, antiplatelet, and antihypertensive medications, as indicated. Exclusion criteria included critical limb ischemia, severe peripheral neuropathy, diabetes mellitus, revascularization for PAD within the prior 3 months, unstable angina or severe coronary artery disease, or other conditions that would prohibit cardiopulmonary exercise (CPX) testing or training. All subjects were sedentary before enrollment. All subjects were informed of testing protocols and the potential risks and benefits of participation. Each subject provided written informed consent before enrollment in the study. Group breakdown was as follows: supervised exercise, n=15; home exercise, n=20. The institutional review boards at Duke University and the University of Colorado approved the research protocols.

**Methods**

Subjects

Subjects were selected from a larger randomized clinical trial of home versus supervised exercise in PAD subjects as shown in Figure 1. Seventy-eight subjects with PAD were randomly assigned and completed baseline testing. During the course of the study, 27 subjects did not complete the protocol. Figure 1 categorized these reasons as due to personal (eg, family, job) or medical (eg, intervention, illness) reasons. Fifty-one subjects completed the protocol; however, 16 of these did not have complete data sets. Figure 1 also lists the reasons for unusable data from those who did complete the protocol. Thirty-five subjects completed the protocol with complete, usable data sets.

Refused F/U muscle biopsy (11)

Subjects who completed exercise training + testing with usable data from biopsy, CPX and exercise training (n = 35)

Data unusable due to:
- Poor CPX (2)
- Histology quality (3)

Subjects who completed the protocol with incomplete or unusable data (n = 16)

During the study 17 dropped out due to medical reasons
- Intervention (3)
- Developed co-morbid illness/medical complications (10)
- CLI (1)
- Plavix Rx (3)

During the study 10 dropped out due to behavioral/personal reasons
- Family/personal issues (7)
- Occupational conflict (1)
- Moved (1)
- Loss to F/U (1)

Figure 1. Flow chart of enrollment and completion of subjects. F/U indicates follow-up; CPX, cardiopulmonary exercise; CLI, critical limb ischemia.

Baseline Testing/Skeletal Muscle Biopsy

All subjects underwent ABI and CPX testing before skeletal muscle biopsy. ABI testing was performed in both legs in the supine position after 10 minutes of rest. The lowest ABI value was used for analysis. All subjects underwent a maximal CPX with a 12-lead ECG and expired gas analysis on a treadmill. Expired gases were analyzed continuously using a ParvoMedics (Sandy, UT) or MedGraphics (St. Paul, MN) unit and averaged in 15-second intervals. The Gardner graded treadmill protocol (performed at a standard speed [2 miles/hour] with a 2% grade increase every 2 minutes) was used for the cardiopulmonary test. Peak VO2 (ml/kg/min) was measured in all patients. Oxygen pulse was acquired by the following equation: Peak VO2 (L/min)/Peak heart rate.

Skeletal muscle biopsies were taken from the medial aspect of the gastrocnemius muscle at rest at least 24 hours after CPX testing. Biopsies were delayed by 24 hours to ensure that the skeletal muscle was in a resting metabolic state. A modified Bergstrom needle technique was used to obtain multiple 20- to 40-mg samples of skeletal muscle following local anesthesia with 2% lidocaine and a 1-cm skin incision. Two samples were snap frozen for citrate and myoglobin analysis. A separate sample was embedded in cross-section using optical cutting temperature tissue freezing medium (Tissue-Tek, Sakura Finetek USA, Inc, Torrance, CA), snap frozen in liquid nitrogen, and stored at −80°C for histological analyses. Muscle biopsies were performed at 0, 3, and 12 weeks in the supervised exercise group and 0 and 12 weeks in the home exercise group.

Exercise Training

PAD subjects randomly assigned to supervised exercise came to medically supervised sessions 3 times a week until 36 sessions were completed. No subject exceeded 16 weeks to complete the 36 sessions. All subjects exercised on a treadmill at the workload at which claudication onset was documented from the baseline CPX. Subjects were asked to exercise to near maximal pain using a standardized claudication scale, at which time the subject stepped off the treadmill and rested until claudication pain subsided. Exercise and rest cycles were repeated during each exercise training session until the accumulation of 30 to 40 minutes of exercise was completed, referring to the actual time walked, not including rest breaks. After a subject was able to walk for 8 to 10 minutes at his or her initial workload, speed and elevation were increased to elicit claudication again. To provide optimal medical care for subjects not randomly assigned to supervised exercise, subjects in the home exercise group were given identical exercise instructions as the supervised group; however, they were asked to perform the exercise training on their own at home with an exercise prescription following the American College of Cardiology–American Heart Association guideline recommendation for PAD exercise training (class 1 level evidence A) at the time of the study according to current recommendations. (Of note, these are also the current recommendations as of publication.) All exercise training for both supervised and home groups was recorded as number of sessions per week and minutes per session. For supervised subjects, this was recorded by an exercise physiologist conducting the training protocol. For the home subjects, patients were asked to complete an exercise diary, which was collected at the end of the 12 weeks.

**Histological Analysis/Indirect Immunofluorescence**

Frozen muscle sections (7-μm thick) were cut using a Leica CM-1950 cryostat and placed on positively charged slides. The slides were stored at −80°C until they were needed. Sections were removed from the freezer and allowed to reach room temperature. Sections were fixed by immersion into 100% ice-cold acetone for 10 minutes, air dried for 10 minutes (room temperature), and then rehydrated in PBS for 5 minutes. Sections were blocked for 30 minutes with 10% normal goat serum in PBS containing 0.5% coldwater fish skin gelatin (Sigma). Endothelial cells were detected using a mouse anti-human CD31 (clone 9G11, 20 μg/mL, R&D Systems, Inc.) followed by goat anti-mouse Alexa Fluor 488 (40 μg/mL) followed by goat anti-mouse Alexa Fluor 568 (10 μg/mL). Two samples were snap frozen for citrate and myoglobin analysis. A separate sample was embedded in cross-section using optical cutting temperature tissue freezing medium (Tissue-Tek, Sakura Finetek USA, Inc, Torrance, CA), snap frozen in liquid nitrogen, and stored at −80°C for histological analyses. Muscle biopsies were performed at 0, 3, and 12 weeks in the supervised exercise group and 0 and 12 weeks in the home exercise group.
µg/mL, Invitrogen) as previously described.19 Hybridoma lines BA-D5 and SC-71 were obtained from the American Type Culture Collection (Manassas, VA).20 Hybridomas were cultured and purified by the Lymphocyte Culture Center at the University of Virginia. BA-D5 (8.9 µg/mL) and SC-71 (12.3 µg/mL) were coincubated overnight at 4°C in blocking solution plus 5% normal mouse serum. Slides were washed twice in PBS, and coverslips were applied using Prolong-Gold (Invitrogen).

Images were captured using a Zeiss LSM 510-UV confocal microscope at a final magnification of ×100. A blinded observer with no knowledge of the group assignment or time point with the group analyzed the images using Image-Pro Plus 4.5.1. Fibers that were unstained by either BA-D5 or SC-71 were counted as type IId/x. CD for each sample was calculated by dividing the total number of CD31-positive capillaries by the muscle fiber area (mm² of tissue, which was measured using Image Pro Plus) per section. Examination of the capillary-fiber ratio were performed on individual fibers (1) that did not touch 2 preselected adjacent boundaries of the image and (2) in which more than 75% of the circumference of the fiber was seen.

Hypereemic Limb Blood Flow
In a subset of subjects (9 supervised exercise and 15 home exercise), calf blood flow was measured in the supine position by venous occlusion strain-gauge plethysmography (DE Hokanson, Issaquah, WA) at rest and during reactive hyperemia immediately after release of 5-minute cuff occlusion, as previously described.21–23 The involved leg (lowest ABI) was supported just above the level of the heart, and a mercury-in-Silastic strain gauge was placed around the widest part of the calf. Before all assessment, an ankle cuff was inflated to 50 mm Hg above systolic pressure for the 60 seconds to eliminate foot circulation from the measurement. A pneumatic cuff was placed on the thigh and inflated to 30 mm Hg to achieve venous occlusion. The cuff occlusion was maintained for several cardiac cycles (4–6 cycles) to obtain resting blood flow measurements. Blood flow was expressed as mL/100 mL of muscle per minute. Resting blood flow was calculated as the average of 5 separate measurements in each limb. Peak reactive hyperemia blood flow was determined after limb ischemia induced by the proximal thigh cuff that was inflated 50 mm Hg above systolic blood pressure for 5 minutes. Postocclusion reactive hyperemia blood flow measurements were made every few seconds, and the highest value achieved was taken as the peak value. Analysis of peak blood flow was taken from 9 supervised exercise and 15 home-exercise subjects.

Measures of Markers of Oxidative Metabolism
In addition to the assessment of fiber type composition by histology, a 25- to 30-mg sample was immediately snap frozen into liquid nitrogen at the time of biopsy, stored at −80°C, and homogenized at 80°C in blocking solution plus 5% normal mouse serum. A 25- to 30-mg sample was immediately snap frozen into liquid nitrogen at the time of biopsy, stored at −80°C, and homogenized at 80°C in blocking solution plus 5% normal mouse serum. A 25- to 30-mg sample was immediately snap frozen into liquid nitrogen at the time of biopsy, stored at −80°C, and homogenized at 80°C in blocking solution plus 5% normal mouse serum.

Table 1. Demographic and Clinical Characteristics of Peripheral Artery Disease Subjects

<table>
<thead>
<tr>
<th></th>
<th>Supervised Exercise</th>
<th>Home Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Age, y</td>
<td>69±10</td>
<td>67±10.1</td>
</tr>
<tr>
<td>Race, % white</td>
<td>80%</td>
<td>75%</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>7/8</td>
<td>11/9</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.3±5.2</td>
<td>26.1±3.9</td>
</tr>
<tr>
<td>ABI</td>
<td>0.63±0.2</td>
<td>0.65±0.1</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Past smoker, %</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>53</td>
<td>60</td>
</tr>
<tr>
<td>ACE/ARB use, %</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Beta blocker use, %</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Antiplatelet use, %</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>Aspirin use, %</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Values are mean±SD or percentages. BMI indicates body mass index; ABI, ankle brachial index; ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

at 405 nm. L (cm) is the path length for absorbance measurement, which was 0.552 cm for the plates used. Citrate synthase activity was analyzed in 11 supervised exercise subjects and in 6 home exercise subjects. In addition, myoglobin levels were measured by ELISA (Calbiotech, Spring Valley, CA). Lysates were diluted 1:8000, and 20 µL of lysate was dispensed in each well (approximately 2.5–25 ng of protein) with the enzyme conjugate reagent and incubated at room temperature for 45 minutes, and the wells were washed with washing buffer. In each well, 100 µL of TMB was dispensed, and the reaction was incubated at room temperature for 20 minutes. The absorbency of samples was read at 450 nm using the Organon Teknika plate reader. The absorbency readings were plotted on a standard curve, giving the myoglobin concentration per sample as ng of myoglobin/ng of soluble protein. Myoglobin was analyzed in 13 supervised-exercise subjects and in 16 home-exercise subjects.

Statistical Analysis
Differences in demographic and clinical characteristics between supervised and home exercise groups were determined using a 1-way ANOVA. For categorical variables, differences were determined by χ² analysis. The primary hypothesis was that with supervised training, CD would increase before any changes in peak VO₂. Subjects randomly assigned to supervised training had 3 time points for assessment: baseline, week 3, and week 12. Therefore, a repeated-measures, mixed-model ANOVA with post hoc testing using the Bonferroni adjustment for multiple comparisons was performed for this analysis. The same analysis was performed between supervised and home groups, where the common time points were baseline and 12 weeks for CD and peak VO₂. For assessment: baseline, week 3, and week 12. Therefore, a repeated-measures, mixed-model ANOVA with post hoc testing using the Bonferroni adjustment for multiple comparisons was performed for this analysis. The same analysis was performed between supervised and home groups, where the common time points were baseline and 12 weeks for CD and peak VO₂. First, change scores after training between groups were analyzed. Second, postraining measurements were tested for differences after controlling for baseline measurements. Bivariate correlations were performed to determine the relationships between CD and the functional variables peak VO₂. Tabular data are presented as means±SD. Probability values of <0.05 were considered significant for all tests.

Results
Patient Demographics and Exercise Adherence
Table 1 shows subject demographics and clinical characteristics; the group represents a typical clinical population with intermittent claudication across all variables. Subjects were well matched for age, race, gender, body habitus, ABI,
medication use, and smoking history between supervised and home exercise groups. ABI did not change throughout the study for either the supervised exercise group (pre 0.62±0.23, post 0.63±0.25) or the home exercise group (pre 0.68±0.19, post 0.69±0.21). Weight did not change throughout the study for either the supervised exercise group (pre 78.3±15.7 kg, post 78.7±15.6 kg) or the home exercise group (pre 77.9±11.9 kg, post 78.4±12.7 kg). The home exercise group trended toward exercising more days per week versus the supervised exercise group (3.0±1.3 versus 2.3±0.3) and more minutes per week (104±59 versus 81±21), but neither difference reached statistical significance.

Changes in CD and Peak VO2 by Type of Exercise
Supervised Exercise Training
Figure 2 shows the changes during supervised exercise training for both CD and peak VO2. Subjects randomly assigned to supervised training had 3 time points for assessment: baseline, week 3, and week 12. For the overall ANOVA model, the probability value was significant at 0.0198. Post hoc testing revealed CD per area had a 31% increase from baseline (0 weeks) to 3 weeks after exercise training (216±66 versus 284±77, P=0.049), whereas peak VO2 was unchanged. After 12 weeks of exercise training, CD (286±77) was virtually identical to the value obtained at the 3 week time point and remained greater than baseline (P=0.04). There was no statistically significant increase in capillary per fiber from baseline to 3 weeks after exercise training (1.72±0.5 versus 1.87±0.7, P=0.44), but it approached significance at 12 weeks after exercise training (1.72±0.5 versus 2.04±0.7, P=0.08). Cross-sectional fiber area was not different between time points in the supervised exercise group in either type I or type II fibers, nor did the cross-sectional area change relate to changes in CD. Although peak VO2 did not show an increase from 0 to 3 weeks (P=0.68), it did increase significantly at 12 weeks compared with 0 weeks (15.3±2.8 versus 16.8±3.8, P<0.01). From these results, it appears that CD changes occur before changes in peak VO2 during supervised exercise training.

Home Exercise Training
CD per area remained unchanged from baseline to 12 weeks in the home exercise group (238±78 versus 235±91, P=0.91). Capillaries per fiber did not significantly increase (1.79±0.15 versus 1.89±0.7, P=0.48). Similarly, peak VO2 remained numerically and statistically unchanged from baseline to 12 weeks (15.9±4.6 versus 15.9±4.7, P=0.99).

Between Group Changes in CD and Peak VO2
Our primary analysis was that the change in peak VO2 for the supervised group was 10.2±13.1% versus 0.0±15.7% for the home group and approached significance (P=0.09). With additional post hoc testing adjusting for baseline peak VO2, the 12-week peak VO2 was significantly different between groups (P=0.03). CD per area approached significance at a probability value of 0.09, as the supervised group showed a robust increase and the home group slightly decreased (Table 2).

Changes in Muscle Measures of Fiber Type, Peak Blood Flow, Oxygen Pulse, and Oxidative Metabolism by Type of Exercise
Fiber type composition (type I, IIA, and IID/x), peak blood flow, and oxygen pulse (as shown in Table 2) did not change

![Figure 2](https://image-url)

**Figure 2.** Supervised exercise training concomitant time course of change for capillary density and peak oxygen uptake (VO2) (●, peak VO2; ■, capillary density). *Significantly different vs 0 weeks, P<0.05.

### Table 2. Pre- and Post-Peak VO2, Skeletal Muscle, and Oxygen Pulse Measures

<table>
<thead>
<tr>
<th></th>
<th>Supervised Exercise</th>
<th>Home Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Wk</td>
<td>3 Wk</td>
</tr>
<tr>
<td>Peak VO2</td>
<td>15.3±2.8</td>
<td>15.1±3.1</td>
</tr>
<tr>
<td>Capillarization (capillaries per mm²)</td>
<td>216±66</td>
<td>284±77*</td>
</tr>
<tr>
<td>Capillary:fiber</td>
<td>1.72±0.5</td>
<td>1.87±0.7</td>
</tr>
<tr>
<td>Fiber type I (%)</td>
<td>59±13</td>
<td>57±20</td>
</tr>
<tr>
<td>Fiber type IIA (%)</td>
<td>21±9</td>
<td>22±10</td>
</tr>
<tr>
<td>Fiber type IID/x (%)</td>
<td>20±13</td>
<td>20±16</td>
</tr>
<tr>
<td>Oxygen pulse (L/min)</td>
<td>10.4±3.2</td>
<td>10.4±3.1</td>
</tr>
<tr>
<td>Peak blood flow (mL/100 mL tissue per min)</td>
<td>7.1±5.2</td>
<td>10.3±5.0</td>
</tr>
</tbody>
</table>

Values are mean±SD. VO2 indicates peak oxygen uptake.
*Indicates difference between 0-wk values in supervised group, P<0.05.
†Indicates that 12-wk values are different between groups after controlling for baseline value, P<0.05.
‡Indicates that change from 0 to 12 wk is different between groups, P<0.05.
in the supervised or home exercise group with training. In addition, analyses were performed on a subset of subjects to look for fold changes in markers of oxidative metabolism specifically, citrate synthase activity and myoglobin from baseline to 12 weeks. In the supervised exercise group, citrate synthase was \(1.10 \pm 0.89\)-fold increased at 12 weeks compared with baseline, and in the home exercise group, citrate synthase was \(1.27 \pm 0.51\)-fold increased at 12 weeks compared with baseline. In the supervised exercise group, myoglobin was \(1.09 \pm 0.74\)-fold increased at 12 weeks compared with baseline, and in the home exercise group, myoglobin was \(0.73 \pm 0.47\)-fold increased at 12 weeks compared with baseline. Thus, there was no significant difference between groups in terms of citrate synthase or myoglobin from baseline to 12 weeks.

**Hemodynamic Changes Between Supervised and Home Exercise Groups After 12 Weeks**

Two measures, oxygen pulse and peak leg blood flow, changed significantly between groups at 12 weeks (Table 2). In both cases, the supervised group showed a modest increase, whereas the home exercise group had a decrease.

**Relationship Between CD and Peak VO\(_2\)**

Before the start of exercise training, there was a significant relationship between CD per area and peak VO\(_2\) \((P<0.02, r=0.412)\) in all subjects, as shown in Figure 3. Following supervised exercise training, there also was a strong relationship between CD per area and peak VO\(_2\) \((P<0.03, r=0.569)\). This relationship did not exist in the home exercise group at the end of the 12 weeks.

**Discussion**

This study was designed to test the hypothesis that increases in CD would occur in calf skeletal muscle before improvements in peak VO\(_2\) following exercise training in PAD. Indeed, after 3 weeks of supervised exercise training, PAD patients showed a significant increase in CD without a concomitant increase in peak VO\(_2\) (Figure 2). However, after 12 weeks of exercise training, peak VO\(_2\) was also shown to increase without a significant additional increase in CD. These results were observed in the absence of changes in muscle oxidative markers, such as fiber type, citrate synthase, or myoglobin. The finding that citrate synthase, which is directly related to mitochondria content, did not increase after exercise training confirms our previous findings and may reflect a more global muscle myopathy associated with PAD.

We are left to infer that the improved peak VO\(_2\) in subjects with PAD following supervised exercise is likely, at least in part, due to the increase in CD that might precede the collateral remodeling that is needed to increase perfusion or that a period of increased perfusion may be necessary for an improvement in mitochondrial function, which may be disturbed in PAD. However, we cannot exclude changes in other unidentified oxidative muscle measures.

The results of this study are worth comparing with what is known to occur in healthy subjects, where 2 to 5 weeks of training induces an increase in peak VO\(_2\). Healthy individuals show an increased peak VO\(_2\) in the early phases of exercise training, likely due predominantly to improved hemodynamics (eg, stroke volume) and to a lesser extent to skeletal muscle adaptations. Furthermore, normal subjects also demonstrate improvements in oxygen pulse following 12 weeks or less of supervised exercise training that was not observed in the our present PAD study. The design of this study allowed for an intermediate time point, 3 weeks, measuring both CD (along with other oxidative markers) and peak VO\(_2\) in the supervised exercise group. A failure to improve peak VO\(_2\) following 3 weeks of exercise training, as witnessed in this study, should be viewed as an abnormal, maladaptive response to exercise training. Oxygen pulse, a surrogate for stroke volume, also failed to increase at this intermediate 3-week time point. It is possible that because claudication is the limiting factor for exercise tolerance in this population, exercise intensities or duration needed to achieve central hemodynamic improvements were not met. Despite limitations inherent in human studies, our data are consistent with the hypothesis that angiogenesis, manifested by an increase in CD in ischemic leg muscle, may be necessary before peak VO\(_2\) can be increased. To our knowledge, this is the first randomized, controlled human trial in subjects with PAD to demonstrate that finding.

In addition to the temporal association of changes in CD and peak VO\(_2\) in subjects with PAD following the onset of exercise, a second important finding was the relationship between CD and peak VO\(_2\) both at baseline in all subjects and following exercise training in the supervised group (Figure 3). Although this relationship exists in cross-sectional data with a wide range of peak VO\(_2\) in "healthy subjects," it has not been demonstrated in a group of subjects with similar peak VO\(_2\) before or after exercise training. However, we did
not show a relationship between the change in CD and the change in peak VO2. Thus, our data are not meant to suggest that angiogenesis in calf skeletal muscle is the sole mechanism that could account for the increases in peak VO2 seen in PAD patients following exercise training.

The third major finding was that despite being given similar exercise prescriptions, only patients in the supervised exercise training group, and not those in the home exercise group, improved peak VO2 and CD. It was hypothesized, on the basis of previous investigations showing the superior improvements in exercise tolerance following supervised versus home training, that the home group would show little, if any, improvement. This hypothesis was correct and again shows the clinical efficacy of a supervised exercise intervention to improve functional capacity in PAD patients. Although when peak VO2 and CD (per area) were analyzed by change scores from 0 to 12 weeks, the differences between groups were close to statistical significance (P = 0.07 and 0.09), they did not reach statistical significance. However, a second analysis comparing the 12-week peak VO2 between groups after controlling for baseline peak VO2 did show a difference (P < 0.05). This observation, along with the acknowledgment that the pre-post peak VO2 in the supervised group improved (P < 0.01) but the home group had no change, demonstrates the superiority of a supervised exercise program in improving functional capacity.

The home exercise group was included as an optimal medical care group, and interestingly, the home exercise group tended to exercise more days per week than the supervised group (3.0 versus 2.3 days, not significant) and did more minutes of exercise per week (104 versus 81 minutes, not significant). However, they did not improve their CD or peak VO2. Through training logs and empirical exit interviews (data not captured), we found that the home exercise subjects rarely exercised as recommended to the point of 3 to 4/5 pain severity of claudication, rested until it was relieved, and then repeated to accumulate their minutes. Instead, despite instruction to the contrary, they exercised at an exercise level well below intolerable claudication, thus failing to achieve the appropriate exercise intensity that allowed the more substantial changes observed in the supervised group. Data from this study, as in previous studies, show that supervised exercise is necessary for patients to achieve maximal benefit demonstrated by improved functional capacity. We now show that supervised exercise is also needed to gain increases in CD. If the home group had done the same intensity exercise (3–4/5 claudication) as the supervised group, we would have expected the results to be similar between groups. Although this was not the design or focus of the study, this is a key concept and supports the importance of supervised exercise versus home exercise for functional and skeletal muscle improvements in PAD. There are likely many reasons that home exercise has less clinical utility than supervised exercise, such as a patient not having access to a treadmill or gym membership, weather limiting outdoor walking, nowhere to rest while walking outside, and a lack of motivation or natural avoidance to exercising in pain. Unfortunately, this study was not designed to determine why home exercise does not yield the beneficial results of supervised exercise; we can only speculate, at best, as to why this occurred in the present study and in previous studies. Future studies, capturing the minutes of exercise in 3 to 4/5 claudication versus <3/5 pain, would be clinically helpful in understanding the mechanism of improvement.

Historically, measurements of hemodynamics by ABI and plethysmography have not proven to be strong predictors of exercise tolerance in PAD.22,33 In our study, the ABI did not change in either group. Our data confirm previous investigations of leg blood flow in that despite a difference in peak blood flow between the supervised and home exercise, the increase in flow in the supervised group did not positively relate to increases in peak VO2. The relevance of microvascularity as measured by CD is that it represents the interface between hemodynamics and skeletal muscle and therefore maybe more important than ABI. Improved CD increases the diffusion potential and blood resident time of the skeletal muscle, thereby improving oxygen extraction, substrate utilization, and oxidative capacity.13,34,35

There are several limitations of this study. First, we used 5 minutes of cuff occlusion during the plethysmography evaluation for posthyperemic blood flow. Although this has been used by others,21–23 this is not a perfect measure of femoral artery endothelial function or true maximum flow measured with absolute minimum vascular resistance. A second limitation is that although we did not detect changes in citrate synthase or myoglobin with supervised exercise, we did not measure all proteins of oxidative metabolism, and we cannot exclude the possibility that other proteins might be regulated in a different manner. Though not a true limitation, it is interesting to note that changes in CD (endothelial cells/mm2) were statistically significantly increased with supervised exercise. The 2 components of that measure (endothelial cells/fiber and the mean area/fiber) changed but not statistically significantly in the expected direction to yield an overall change in capillaries/mm2. Specifically, following supervised training, endothelial cells per fiber were numerically but not statistically greater, and fiber area was numerically but not significantly lower. We did not power the study to detect these differences, and additional studies would be needed to determine whether one of these processes occurred first or was dominant in the angiogenic response we observed in subjects with PAD. A third limitation was that we did not prospectively collect detailed data in the home exercise training logs. We collected total number of days and total number of minutes exercised in the home exercise group, but, unlike for the supervised group, we do not have accurate data on whether that exercise induced claudication at all or for how long. Future studies are needed to test the hypothesis that the reason that supervised exercise resulted in angiogenesis in ischemic muscle and increases in peak VO2 in patients was due to presence and duration of exercise-induced claudication.

In summary, the results of this study show a temporal dissociation between the timing of increased CD and the increased peak VO2 in patients with PAD treated with supervised exercise, such that CD increased before a significant increase in peak VO2 occurred. The results also show a relationship between CD and peak VO2 both before and after
supervised exercise training; such findings have not been reported in healthy normal subjects. These findings may suggest that it is necessary to increase microvasculature to improve exercise capacity in PAD patients. This is further supported by the lack of improvement in other skeletal muscle markers of oxidative capacity and by the maladaptive response to peak VO2 and oxygen pulse compared with healthy subjects. Therefore, on the basis of these results, it is concluded that angiogenesis by supervised exercise training is an important mediator for improving peak VO2 in PAD patients.

Sources of Funding
This project was supported by R01-HL755752 from the National Institute of Health, National Heart, Lung, and Blood Institute and the American Heart Association for Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation.

Disclosures
None.

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Angiogenesis in Skeletal Muscle Precede Improvements in Peak Oxygen Uptake in Peripheral Artery Disease Patients


Arterioscler Thromb Vasc Biol. 2011;31:2742-2748; originally published online August 25, 2011;
doi: 10.1161/ATVBAHA.111.230441

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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