Exercise in Patients with Intermittent Claudication Elicits Signs of Inflammation and Angiogenesis

U. Palmer-Kazen\textsuperscript{a,b,*}, P. Religa\textsuperscript{c}, E. Wahlberg\textsuperscript{a,b,d}

\textsuperscript{a} Department of Molecular Medicine and Surgery, Vascular Biology Laboratory, Karolinska Institute, Stockholm, Sweden
\textsuperscript{b} Department of Vascular Surgery, Karolinska University Hospital, Stockholm, Sweden
\textsuperscript{c} Department of Angiogenesis Research, MTC, Karolinska Institute, Stockholm, Sweden
\textsuperscript{d} The Heart Center, Linköping University Hospital, Linköping, Sweden

Submitted 14 January 2009; accepted 6 August 2009
Available online 23 September 2009

\textbf{KEYWORDS}
Claudication; Angiogenesis; Arteriogenesis; Exercise; Growth factors

\textbf{Abstract}

\textbf{Objectives:} Previous studies have demonstrated elevation of systemic levels of inflammatory cytokines after treadmill exercise in patients with intermittent claudication (IC), but it is unknown if growth factor expression also is stimulated. The aim of this study was to assess whether physical exercise-induced ischemia elicits an inflammatory response and increase in local and systemic vascular growth factor expression in patients with IC.

\textbf{Methods:} Nineteen patients with IC had plasma concentrations of inflammatory markers (IL-6, TNF-alpha, hs-CRP) and vascular growth factors (VEGF and FGF-2) measured before and at four time points after a treadmill exercise test. In 10 patients a gastrocnemius muscle biopsy was obtained to measure VEGF and FGF-2 mRNA. Plasma concentrations of vWF were also measured. Five patients who underwent the treadmill test without experiencing calf pain were enrolled as controls.

\textbf{Results:} Plasma concentrations of IL-6 increased after exercise ($p = 0.004$), while TNF-alpha and hs-CRP were unchanged ($p = 0.191$ and $p = 0.709$, respectively). Plasma concentrations of VEGF were similar ($p = 0.151$) at the different time points after exercise but FGF-2 levels decreased ($p = 0.013$). In biopsies after treadmill testing VEGF-A mRNA was increased ($p = 0.043$), but no change was observed for FGF-2 ($p = 0.456$).

\textbf{Conclusion:} Exercise in IC triggers an inflammatory response as exemplified by elevated concentrations of IL-6. After exercise-induced pain, VEGF mRNA in calf muscle is increased. Therefore, it is plausible that angiogenesis is stimulated by exercise in IC.

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\textbf{Introduction}

Peripheral arterial disease (PAD) is common and affects about 20% of the population above the age of 60, and 7% have intermittent claudication (IC).\textsuperscript{1} PAD, including IC, is
a strong marker for future cardiovascular events and increased mortality.\(^2\) Several studies have demonstrated that hemostatic markers as D-dimer and thrombin-antithrombin levels are elevated systemically in patients with PAD compared to controls,\(^3,4\) and more recently increases in inflammatory and endothelial biomarkers have been observed.\(^5\)

While pharmacological risk reduction is mandatory, invasive procedures to reduce symptoms are not indicated for most IC patients. Physical exercise is the primary treatment strategy, but the mechanisms explaining why exercise therapy is beneficial are unknown. Adaptation and improvement of the energy metabolism in the muscle is probably most important, and to a lesser extent (around 30\%) blood flow improvement contributes.\(^6,7\) Collateral growth, arteriogenesis, may be a process that enhances blood flow when the patient exercises. Its development is rather complex involving several steps such as increases in shear stress against the vessel wall, production of cytokines, a local vessel wall inflammation and finally remodeling and enlargement of the arterioles into large collateral arteries.\(^8,9\) Besides arteriogenesis formation of new capillaries, angiogenesis, may also be of importance for improvement of tissue perfusion following exercise in IC. Hypoxia, the most important trigger for angiogenesis, may be involved in IC patophysiology, i.e. when the pain forces the patient to stop walking. This hypoxic initiation of angiogenesis involves production of vascular endothelial growth factor (VEGF).\(^10,11\) However, patients with chronic PAD have very low levels of VEGF in distal muscle compared to proximal parts of the leg.\(^12\) In patients with chronic leg ischemia and ischemic heart disease, elevated systemic levels of fibroblast growth factor-2 (FGF-2) have been reported.\(^13,14\) It is not known whether growth factor expression is stimulated in IC patients reaching the absolute claudication distance (ACD).

Better knowledge of endogenous arteriogenesis and angiogenesis in PAD may lead to understanding of the effects of exercise therapy and will impact development of new therapeutic strategies. Accordingly, the aim of this study was to assess whether physical exercise-induced ischemia will cause an inflammatory response and local vascular growth factor expression that may play a role for these two reparative processes in patients with IC.

Materials and methods

Study population

Participants were recruited from consecutive patients referred to the Vascular Surgery Department for evaluation of PAD. Inclusion criteria were strict; ankle-brachial index (ABI) between 0.5 and 0.8 and a reproducible ACD, defined as <20\% difference in two consecutive treadmill tests, and ACD had to be between 1 and 6 min. Patients with diabetes mellitus, severe heart failure, and myocardial infarction within 6 months or unstable angina, neoplastic, immunologic or chronic inflammatory diseases were excluded. A large number of patients were screened using medical records and interviews, and during the treadmill tests further patients were excluded. Accordingly, the patients were not representative for IC patients in general and selected in order to identify a group with reproducible walking distances and as little concomitant diseases as possible.

Six screened patients, who fulfilled all criteria with one exception — they had ACD longer than 6 min and walked for a mean of 12 min (range 10–16 min) on two consecutive treadmill tests served as a control group with the purpose to enable evaluation of cytokine expression after exercise in patients without muscle pain. Evaluation of data from this group was considered exploratory only and they did not participate in the biopsy substudy.

The hospital’s Ethics committee approved the study protocol. All patients gave written consent to participate after receiving oral and written information.

Study procedure

Patients visited the hospital on a separate day to have ABI measured and to perform qualifying treadmill tests. The treadmill was set at 3.2 km/h and a slope of 14\% was used. Included patients returned a few days later for the main investigations. At that time resting baseline measurements (ABI, blood samples, and biopsies when applicable) were performed 1 h before the exercise test. The patients then walked on the treadmill until ACD, rested 5 min, and performed another test until ACD. After walking, ABI was again measured within 2 min and blood samples drawn at 15, 60 and 120 min afterwards. The levels of high sensitivity CRP (hs-CRP), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-alpha) and the vascular growth factors VEGF and FGF-2 were analyzed at all these time points. A second biopsy was to obtain 120 min after the test in some of the patients. The time points selected for investigation were based on available literature covering exercise and cytokine expression as well as our hypotheses on possible mechanisms of action.\(^15–17\)

Blood sample analysis

Blood samples were drawn from the antecubital vein and plasma separated by centrifugation within 30 min. Plasma was frozen for later analysis of vWF (LIA-test), hs-CRP (using particle enhanced immunonephelometry), IL-6 and TNF-alpha samples (both by ELISA). Growth factors samples were centrifuged within 2 h at 2000 \( \times \) \( g \) for 20 min, the plasma separated and stored at \(-80^\circ C\) for subsequent analysis. The concentrations of total human VEGF and FGF-2 in plasma were determined using ELISA (Quantikine\(^{®}\) High Sensitivity human FGF basic Cat no HSFB75 and Quantikine\(^{®}\) human VEGF Cat no DVE00, R&D Systems, Minneapolis, MN). Each analysis included a standard solution of VEGF or FGF-2 and all standards and samples were run in duplicate. All techniques and materials were used according to the manufacturer’s instructions.

Microbiopsy procedure

Ten of the patients underwent percutaneous muscle biopsies before and after the test. A spring-loaded, reusable instrument, Bard\(^{®}\) Magnum\(^{®}\) Biopsy Instrument, kindly provided by BARD (BARD Norden AB, Helsingborg, Sweden)
was used. This device is effective for fine needle percutaneous biopsies of various tissues\textsuperscript{18} and has recently been evaluated for skeletal muscle in a validation study\textsuperscript{19} by comparing it to needle biopsy according to Bergstrom. Core tissue biopsy needles, 12 Gauge (Bard\textsuperscript{R} Magnum\textsuperscript{R}, MN1210, BARD), with a sampling notch length of 19 mm and a penetration depth of 15 mm was used.

Under local anaesthesia (2 ml of lidocaine 10 mg/ml), the skin was incised 20 cm medially below the knee using a scalpel and the biopsy needle inserted until the fascia was pierced. The muscle sample was obtained in the gastrocnemius muscle by activation of a trigger-switch that protrudes the needle. The biopsy needle was then pulled out and the sample immediately frozen on dry ice. The needle was inserted one to two additional times through the same incision using other directions to obtain sufficient amounts of tissue (10–15 mg). The procedure was repeated after the exercise test using a new puncture site placed 1 cm from the first one. All biopsies were performed by the same investigator (UPK).

**mRNA expression**

Total RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was obtained using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to protocols of the kits. cDNA were amplified by real-time PCR with primer and probe pairs to human VEGF-A and FGF-2. GCCCACTGAGGAGTCCAACA and TCTCTATGTGCTGGCCTTGGT were used as primers for VEGF-A and CACCATGCAGATTATGCGGATCAAACC as probe (FAM labeled) and TGTGTCTATCAAAGGAGTGTGTGCTA and TCCGTAACACATTTAGAAGCCAGTA were used as primers for FGF-2 and CCGTTACCTGGCTATGAAGGAAGATGGAAG as probe (FAM labeled). Predeveloped human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used to normalize for RNA loading (Applied Biosystems). The reactions were performed using TaqMan Universal PCR, Master Mix (Applied Biosystems, Foster City, CA) in multiplex using an ABI 7700 Prism Sequence Detection System (Perkin Elmer). The cycle was configured as follows: incubation (95 °C, 10 min), 50 cycles of denaturation (95 °C, 15 s), and annealing/extension (60 °C, 60 s). The PCR was performed in multiplex for studied gene and 18S rRNA as internal, endogenous control. This technique amplifies studied gene and internal control in same tube. External relative standard curves were prepared by serial dilution of cDNA from patients with highest expression of VEGF-A and bFGF. Each run consisted of standards and a negative control without template.

**Statistics**

Results were expressed as median and range. Analysis of variance (ANOVA) was used to compare data over time for IL-6 and TNF-alpha. For post-hoc comparisons, Scheffé's test was applied. Paired Student’s t-test between two dependent groups was calculated for comparisons of ABI and vWF before and after exercise. When not normally distributed, Wilcoxon signed-rank test (VEGF-A and FGF-2 mRNA) or Friedman ANOVA by ranks (hs-CRP, VEGF and FGF-2 in plasma) was used. The mRNA analysis was considered as exploratory and we hypothesized that reaching ACD would increase mRNA levels in every patient. No proper power analysis was performed due to lack of previous data. In some patients mRNA levels were below detectable levels and in the non-parametric statistical analysis levels were then considered to be zero. Statistical analysis was performed using Statistica StatSoft software and for all analyses p-values <0.05 were considered statistically significant.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics of patients with intermittent claudication enrolled in the study.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient characteristics</td>
<td>Main group (treadmill ACD &lt; 6 min)</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
</tr>
<tr>
<td>Age (years, mean, SD)</td>
<td>69.1 (53–85)</td>
</tr>
<tr>
<td>Sex: F:M (N)</td>
<td>11:8</td>
</tr>
<tr>
<td>Smoking status (N):</td>
<td></td>
</tr>
<tr>
<td>yes — current</td>
<td>4</td>
</tr>
<tr>
<td>no — stopped &gt;1 year ago</td>
<td>13</td>
</tr>
<tr>
<td>no — never</td>
<td>2</td>
</tr>
<tr>
<td>Hypertension (N)</td>
<td>11</td>
</tr>
<tr>
<td>Coronary artery disease (N)</td>
<td>5</td>
</tr>
<tr>
<td>Drug treatment at inclusion (N):</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>11</td>
</tr>
<tr>
<td>Statins</td>
<td>10</td>
</tr>
<tr>
<td>Duration of claudication (N):</td>
<td></td>
</tr>
<tr>
<td>&lt;3 months</td>
<td>4</td>
</tr>
<tr>
<td>6 months–1 year</td>
<td>3</td>
</tr>
<tr>
<td>&gt;1 year</td>
<td>12</td>
</tr>
<tr>
<td>Medical history walking distance (meters, median, min–max)</td>
<td>250 (50–1000)</td>
</tr>
<tr>
<td>Bilateral leg problems (N)</td>
<td>4</td>
</tr>
</tbody>
</table>

ACD, absolute claudication distance.
Results

Study population

Nineteen patients IC were included in the main group of whom 10 participated in the muscle biopsy substudy. The exploratory control group consisted of only five patients because one patient withdrew from the study. None of the patients experienced any severe inconveniences during or after the treadmill test or biopsy. Their demographic and clinical characteristics are presented in Table 1. There was a significant reduction in ankle-brachial index after treadmill exercise in both groups (Fig. 1).

Plasma levels of inflammatory parameters

Plasma levels of IL-6 increased after the treadmill test ($p = 0.005$; Fig. 2). TNF-alpha and hs-CRP were similar post-exercise ($p = 0.191$ and $p = 0.709$, respectively, data not shown). Five of the patients in the main study group
(26%) had baseline hs-CRP concentrations above 2 mg/L set as the normal upper limit by our hospital laboratory. A small but significant ($p < 0.034$) decrease was noted for vWF (Fig. 3). In the control group the plasma concentrations of IL-6 showed a similar pattern as in the patients reaching ACD with an increase after the treadmill exercise (Fig. 2).

**Plasma levels of VEGF and FGF-2**

There were large individual variations in systemic growth factor levels at base line. VEGF concentrations were similar ($p = 0.151$) at the different time points (Fig. 4A) but for FGF-2 a decrease ($p = 0.013$) was observed after the treadmill test (Fig. 4B).

**Skeletal muscle mRNA levels**

Real-time PCR analysis of VEGF-A ($N = 10$) mRNA demonstrated a 19% ($p = 0.043$) increase in expression 120 min after exercise as compared with pre-exercise levels (Fig. 5) and for FGF-2 mRNA no change ($p = 0.456$) was observed (not shown). In one patient a biopsy had VEGF-A mRNA concentrations below the limits of detection, and five patients had amounts of FGF-2 mRNA below the limits of detection in either one or both of the samples.

**Discussion**

The principal finding in this study is that exercise to claudication on a treadmill results in increased plasma IL-6, while TNF-alpha and hs-CRP remained unchanged. Furthermore, treadmill exercise reaching ACD causes a decrease in plasma FGF-2 and an increased expression of VEGF-A mRNA in calf muscle biopsies.

There is substantial evidence in the literature that inflammation is involved in arteriogenesis and angiogenesis occurring during limb ischemia but published data suggesting a systemic inflammation in PAD has several other more plausible explanations than these two processes. It may for instance, be attributed to generalized atherosclerosis and plaques releasing cytokines into plasma. In line with this a recent study reported higher levels of CRP and vWF in PAD patients than in healthy controls. CRP concentrations were not influenced by exercise in the patients included in this study, a finding supported in the literature. The potential release of CRP from plaques is probably not affected by this exercise provocation. In contrast, vWF did appear to be released from endothelial cells or platelets during or following exercise. Plasma concentrations are reported to remain elevated for up to 60 min after exercise and the rise in vWF in response to exercise is greater in healthy subjects. However, we observed a decrease in vWF following exercise, as have other groups. The vascular pathophysiology in PAD may explain this contradiction. Changes in shear forces during exercise are probably not strong in vascular beds distal to an arterial occlusion, and the damaged endothelial cells in the vasculature of PAD patients are likely to have a reduced ability to release vWF. Our protocol of walking to ACD twice may have caused an extensive release of vWF that led to depletion in the parts of the vascular bed effected by exercise-dependent changes in shear forces.

TNF-alpha concentrations have been reported to rise after exercise in PAD patients, but this was not observed here, perhaps due to the study design. However, we did demonstrate increased concentrations of IL-6 in plasma after exercise at the later time points, a finding that is supported by a previous study. IL-6 could either have been released from skeletal muscle or from locally activated monocytes in the ischemic calf. It maybe that...
inflammation in the vessel wall of collateral arteries, induced by increases in shear stress, contributed to our findings.30

One of the most potent stimulants of angiogenesis is hypoxia, a metabolic state known to occur in the calf of IC patients when reaching ACD.31 Hypoxia upregulates HIF-1 alpha, which stimulates the transcription of the VEGF gene.32 Hence, transcription of VEGF mRNA is upregulated by exercise in both healthy individuals and patients with heart failure.11,33 We observed that calf muscle VEGF mRNA concentrations increased after exercise to claudication and we infer that either transcription was upregulated or the mRNA was stabilized. The local increase in mRNA was not reflected in the plasma levels of this protein during the short time period studied. This absence of an increase of plasma VEGF-A concentration after exercise to claudication has been noted previously.34

Although FGF-2 is a locally acting growth factor that is not circulated and its biological actions stem from its release from storage sites within the extracellular matrix and subsequent local interaction with surface receptors of endothelial cells rather than modification in the total content.35 Continuously elevated plasma concentrations of FGF-2 have been observed in patients with PAD.12 Higher FGF-2 levels are found in distal ischemic muscle samples from patients with PAD compared to proximal non-ischemic parts of the same leg.14 Few patients in our cohort had measurable levels of FGF-2 in skeletal muscle, and the mRNA data was unreliable. However, we observed a clear decrease in plasma FGF-2 concentrations after exercise to claudication. One possible explanation of this observation would be that binding of FGF-2 to extracellular matrix increase as a result of the exercise provocation test.

Figure 4  Box plots (median and quartiles) of VEGF-A (A) and FGF-2 (B) protein concentrations in plasma before, 15, 60 and 120 min after treadmill exercise in main patient group. No difference was observed for VEGF over time (p = 0.151), but FGF-2 levels decreased significantly (p = 0.013, Friedman ANOVA by ranks).
The local and systemic consequences of exercising to ACD for IC patients largely are uncharted. Whilst our findings may not be generalisable to all IC patients, they add weight to the possibility that exercise provokes the release of IL-6 from either atherosclerotic lesions or, skeletal muscle or an activated endothelium. Hypoxia in the calf caused by the walking test leads to increased concentrations locally of VEGF, suggesting initiation of angiogenesis in IC when walking to ACD.

Acknowledgements

The support from research nurse Maritha Johansson for this study is very much appreciated.

Conflict of Interest

EW has received independent research grants from Pfizer AB, Sanofi-Aventis AB and Shering AG for other studies and has been a speaker and a member of advisory boards for several other companies. UPK and PR have nothing to declare.

Ethical Approval

The study was approved by the local ethics committee in Stockholm (Dnr 03-122 and Dnr 2005/948-31).

Funding

Research Grants from the Swedish Research Council, Swedish Heart and Lung Foundation and King Gustaf V:s and Queen Victoria’s foundation supported this study. Eric Wahlberg receives salary support from the Swedish Heart and Lung Foundation.

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