Potential Vascular Mechanisms of Ramipril Induced Increases in Walking Ability in Patients With Intermittent Claudication

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Rationale: We recently reported that ramipril more than doubled maximum walking times in patients with peripheral artery disease with intermittent claudication. Ramipril induced a 123% (range, 103%–142%) increase in maximum walking time when administered for 24 weeks.

Objective: Our aim was to conduct exploratory analyses of the effects of ramipril therapy on circulating biomarkers of angiogenesis/arteriogenesis, thrombosis, inflammation, and leukocyte adhesion in patients with intermittent claudication.

Methods and Results: One hundred sixty-five patients with intermittent claudication (mean, 65.3 [SD, 6.7] years) were administered ramipril 10 mg per day (n=82) or matching placebo (n=83) for 24 weeks in a randomized, double-blind study. Plasma biomarkers of angiogenesis/arteriogenesis (vascular endothelial growth factor-A, fibroblast growth factor-2), thrombosis (D-dimer, von Willebrand factor, thrombin-antithrombin III), inflammation (high-sensitivity C-reactive protein, osteopontin), and leukocyte adhesion (soluble vascular cell adhesion molecule-1, soluble intracellular adhesion molecule-1) were measured at baseline and 24 weeks. Relative to placebo, ramipril was associated with increases in vascular endothelial growth factor-A by 38% (95% confidence interval [CI], 34%–42%) and fibroblast growth factor-2 by 64% (95% CI, 44–85%; P<0.001 for both), and reductions in D-dimer by 24% (95% CI, −30% to −18%), von Willebrand factor by 22% (95% CI, −35% to −9%), thrombin-antithrombin III by 16% (95% CI, −19% to −13%), high-sensitivity C-reactive protein by 13% (95% CI, −14% to −9%), osteopontin by 12% (95% CI, −14% to −10%), soluble vascular cell adhesion molecule-1 by 14% (95% CI, −18% to −10%), and soluble intracellular adhesion molecule-1 by 15% (95% CI, −17% to −13%; all P<0.001).

With the exception of von Willebrand factor, all the above changes correlated significantly with the change in maximum walking time (P=0.02–0.001) in the group treated with ramipril.

Conclusions: Ramipril is associated with an increase in the biomarkers of angiogenesis/arteriogenesis and reduction in the markers of thrombosis, inflammation, and leukocyte adhesion. This study informs strategies to improve mobility in patients with intermittent claudication.


Key Words: biological markers inflammation peripheral arterial disease thrombosis

We have recently demonstrated that treatment with ramipril significantly improves the walking ability and physical functioning aspects of quality of life in patients with peripheral artery disease (PAD) who have intermittent claudication. Ramipril induced a 123% (range, 103%–142%) increase in maximum walking time when administered for 24 weeks. Understanding the mechanisms contributing to this improvement in functional capacity may identify important targets for improving the medical management of intermittent claudication.

Angiotensin-converting enzyme (ACE) inhibitor therapy may mediate improved functional capacity through multiple mechanisms, including increased peripheral blood flow and adaptations in skeletal muscle structure and function. Elevated blood flow may be mediated via vasodilatation through reduction in angiotensin II (Ang-II), sympathetic inhibition, improvement in endothelial function through preservation of bradykinin (BK), and formation of new collaterals (arteriogenesis) or muscle capillaries (angiogenesis). There is substantial evidence to support that both the renin–angiotensin and BK pathways play a crucial role in the regulation of arteriogenesis and angiogenesis through the modulation of several angiogenic factors, including...
vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factor-2 (FGF-2). The activation of the renin–angiotensin system through increased production of Ang-II is also closely related to local vascular inflammation. Ang-II functions as a potent inflammatory stimulus and upregulates the expression of many redox-sensitive cytokines, chemokines, and growth factors involved in the pathogenesis of atherosclerosis, including C-reactive protein (CRP) and osteopontin (OPN). Ang-II also modulates vascular inflammation by regulating the expression of adhesion molecules such as soluble intracellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1), leading to leukocyte activation and recruitment. Furthermore, a growing body of evidence indicates that a prothrombotic state can be induced by both the renin–angiotensin and BK systems, through nitric oxide (NO) and downstream effects on proteins involved in, stimulated by, or associated with thrombosis, including fibrinogen, D-dimer, von Willebrand factor (vWF), and thrombin-antithrombin III (TAT) complex.

Thus, it has been suggested that ACE inhibitors may favorably modify inflammation and thrombosis, both of which are critical in the complications of atherosclerosis and functional decline in patients with PAD. In the current study, we conducted exploratory analyses of the potential mechanisms underlying the improvement in functional capacity observed after ramipril therapy, through the assessment of circulating biomarkers related to angiogenesis/arteriogenesis, inflammation, leukocyte adhesion, and thrombosis.

**Methods**
This investigation is a substudy of a previous trial for which primary end points were reported in February 2013. Whereas the original study involved 3 participating centers, the current substudy was restricted to a single site (165 participants recruited from the Alfred Hospital, Melbourne, Australia). In the 47 participants recruited from the Townsville Hospital and Royal Women’s Brisbane Hospital, Australia, blood collection for exploratory end points was not performed.

**Participant Identification**
The institutional review board of the Alfred Hospital approved the protocol. Participants provided written informed consent, and the study was performed in accordance with the Declaration of Helsinki 2000. Data collection and interventions were performed at the Alfred Hospital between May 10, 2008, and August 23, 2011. Of the 165 patients completing the study, 105 were from general practice clinics in the metropolitan area of Melbourne, 22 from among patients diagnosed with PAD in the noninvasive vascular laboratory of the Alfred Hospital, and 38 participants were recruited from newspaper advertisements.

**Inclusion and Exclusion Criteria**
Inclusion criteria were ankle brachial index (ABI) <0.9 at rest in at least 1 leg, history of intermittent claudication (unilateral or bilateral, as defined by the Edinburgh Claudication Questionnaire and after clinical examination), which was stable for the previous 6 months, and a stable medication regime for 26 months. Exclusion criteria were resting brachial blood pressure ≥160/100 mm Hg, use of either ACE inhibitors or angiotensin receptor blockers currently, or within the previous 6 months, use of potassium-sparing diuretics or potassium supplements currently or within the previous 6 months, renal failure (serum creatinine >0.2 mmol/L), renal artery stenosis, previous coronary or lower extremity revascularization procedures, myocardial infarction in the previous 3 months, major surgery planned during the following year, critical limb ischemia (ischemic ulcer[s] or minor gangrene), and any condition other than PAD that limits the walking ability, including coronary artery disease, chronic obstructive pulmonary disease, and musculoskeletal conditions (assessed during physical examinations and medical history performed by the study physician).

**Randomization and Masking**
Patients were randomly assigned to receive either ramipril (Ramace; Sanofi-Aventis; 10 mg per day for 24 weeks) or matching placebo in a parallel group, double-blind design as previously described. Once the randomization list was generated by the Epidemiology and Preventive Medicine Department of the Alfred Hospital, it was forwarded to the Alfred Hospital Clinical Trials Pharmacy, which prepared and supplied the medication. None of the investigators had access to the randomization list. Ramipril and placebo tablets were identical and supplied in consecutively numbered drug packs. Each patient was assigned a randomization number and received tablets from the corresponding pack. Investigators and patients were blinded to drug assignment. Investigators did not have access to baseline data when they performed follow-up measurements, and patients were not asked which treatment they thought they were receiving. No patients assigned to placebo crossed over to ramipril, or vice versa, during the trial.

**Treadmill Test**
Pain-free and maximum walking times were assessed by a standard treadmill exercise test performed at a speed of 3.2 km/h and a grade of 12%. All patients had a single baseline treadmill test, and assessment was performed blinded to treatment assignment as previously described.

**ABI Measurement**
ABI was measured and calculated by the same investigator for all patient visits, as previously described.

**Duplex Ultrasoundography**
Scanning was performed by a single, qualified, experienced vascular technologist who was blinded to clinical data and treatment assignment as previously described. All images were also assessed and analyzed by 2 independent, experienced vascular physicians blinded to patient identity and treatment (κ>0.94 for interobserver reliability). Peak systolic velocity in lower limb vessel segments was measured to determine the grade of stenosis as previously described. In 111 patients with femoral disease (50 on placebo and 61 on ramipril), we also determined volume flow. Femoral disease was classified as ≥50% stenosis but no occlusion in the common or superficial femoral arteries. Volume flow was calculated from the lumen cross-sectional area and the integrated mean velocity in the common femoral artery 5 cm proximal to the site of stenosis.

**Blood Collection**
A 21-gauge butterfly needle was inserted into an antecubital vein, and the tourniquet was removed immediately. Blood from participants was collected into appropriate anticoagulant tubes and plasma...
immediately separated by centrifugation at 3000 rpm for 20 minutes in a refrigerated centrifuge. Blood was processed and stored at −80°C <60 minutes of collection.

**Laboratory Analysis**

The plasma biomarkers studied can be broadly classified into 4 categories: angiogenic markers (VEGF-A and FGF-2), thrombosis markers (D-dimer, vWF, and TAT), inflammatory markers (high-sensitivity CRP [hsCRP] and OPN), and leukocyte adhesion markers (sVCAM-1 and sICAM-1).

Serum Ang-II levels were assessed by ELISA (Phoenix Pharmaceuticals, Burlingame, CA). Serum VEGF-A and FGF-2 concentrations were also assessed by ELISA (Quantikine human VEGF; R&D Systems, MN). The assay exhibits no significant crossreactivity with other angiogenic factors and has a sensitivity of 7.0 and 9.0 pg/mL, respectively. The assay recognizes human VEGF165 and VEGF121. VEGF165 is the predominant human isoform compromising ≈90% of VEGF-A in blood17 and is also most potent in terms of stimulating angiogenesis.18 VEGF165 and VEGF121 interactions with their receptors (VEGFR1 and VEGFR2) and nonsignaling neuropilin-1 coreceptor have been extensively studied using validated computational models in the context of angiogenesis,17,19–22 including models relevant to PAD.19,22,23 These models have demonstrated that in the absence of neuropilin-1, VEGF165 and VEGF121 behave similarly. When present, however, neuropilin-1 enhances the binding of VEGF165 to VEGFR2, which is a major mediator of the angiogenic effects of VEGF-A.24

D-dimer was measured with a commercial assay (Technoclone, Vienna, Austria). Serum vWF and TAT levels were evaluated with the use of the IMMUBID vWF ELISA (American Diagnostica). hsCRP measurements were performed using an Architect ci16200 latex-enhanced immunoturbidimetric assay (Abbott Diagnostics, Abbot Park, IL). OPN concentrations were assessed using ELISA according to the manufacturer’s instructions and expressed as nanograms per milliliter (R&D Systems).25,26 sVCAM-1 and sICAM-1 levels were also determined by commercially available ELISA kit (R&D Systems). The limits of detection of sVCAM-1 and sICAM-1 were 0.35 mg/mL and 0.60 ng/mL, respectively, and plasma samples were diluted to ensure all samples fell below these limits. The interassay coefficients of variation for all the above assays using 2 levels of control materials ranged from 2% to 7%.

**Statistical Analyses**

All analysts were blinded to treatment assignment. Baseline characteristics were compared using the χ2 test for categorical variables and 1-way ANOVA for continuous variables. We compared 24-week changes from baseline in all parameters by using an analysis of covariance model with terms for treatment and baseline values. Data were expressed as mean (SD) or 95% confidence intervals (CIs). The Pearson correlation coefficient was used for univariate analysis comparing the change in maximum walking times with the change in biomarkers in the ramipril-treated group only. All correlation analyses were adjusted for age, sex, body mass index, diabetes mellitus, hypertension, ABI, cigarette smoking, and medication use. Statistical analysis was performed using SPSS (version 12.0). A P value <0.05 was deemed significant. For the 165 participants who completed the 6-month follow-up, no data were missing for any clinical variable measured.

**Results**

Of 640 potential participants who were approached for recruitment, 45 declined and 425 met an exclusion criterion, leaving 170 eligible participants (Figure 1). Of these, 5 participants withdrew, leaving 165 participants who completed the 6-month follow-up (Figure 1). The ramipril (n=82) and placebo (n=83) groups were similar in age, other cardiovascular risk factors, medication use, and PAD severity, as evidenced by clinical symptoms (walking times) and resting ABI (Table 1). Furthermore, the groups did not differ in creatinine levels at baseline or after ramipril therapy (Table 2), indicating no confounding effects of renal function. Finally, all plasma biomarkers examined were similar in the placebo and ramipril groups at baseline (Table 2).

**Treadmill Test**

Relative to placebo, ramipril was associated with an 82-second (95% CI, 65–99 seconds) increase in mean pain-free walking time (P<0.001) and a 273-second (95% CI, 229–317 seconds) increase in maximum walking time (P<0.001; Table 3). When

[Figure 1. Study flow. PAD indicates peripheral artery disease.]
change in mean arterial blood pressure was included as a covariate, the effects on walking times were shown to be independent of the small change in blood pressure after ramipril treatment (systolic: −2.7 [95% CI, −3.2 to −2.4] mm Hg; P < 0.01; diastolic: −3.0 [95% CI, −3.4 to −2.6] mm Hg; P < 0.01).

**Duplex Ultrasonography**

In 111 patients with femoropopliteal disease (50 on placebo and 61 on ramipril), we determined volume flow in the common femoral artery at both the site of stenosis and 5 cm proximal to the site of stenosis in the leg with lower ABI (limiting leg). Volume flow was unaltered at the stenotic site both in the placebo and ramipril groups. However, relative to placebo, there was a significant increase in volume flow after ramipril therapy in the common femoral artery 5 cm proximal to the site of stenosis (63 [95% CI, 55–71] mL/min; P < 0.0001; Table 3).

**Blood Parameters**

At baseline, there was no significant difference between the placebo and ramipril groups for any of the blood parameters measured (Table 2). Relative to placebo, ramipril was associated with a 29% [95% CI, 26–32%] reduction in Ang-II levels, demonstrating the efficacy of ramipril to inhibit ACE. Relative to placebo, the parameters associated with angiogenesis/arteriogenesis were increased with ramipril. VEGF-A increased by 38% [95% CI, 34–42%] and FGF-2 increased by 64% [95% CI, 44–85%; P < 0.001 for both; Table 2]. With regard to thrombosis markers, relative to placebo, ramipril was associated with reductions in D-dimer by 24% [95% CI, −30% to −18%], vWF by 22% [95% CI, −35% to −9%], and TAT by 16% [95% CI, −19% to −13%]. Inflammatory and leukocyte adhesion markers were also reduced by ramipril relative to placebo: hsCRP by 13% [95% CI, −14% to −9%], OPN by 12% [95% CI, −14% to −10%], sVCAM-1 by 14% [95% CI, −18% to −10%], and sICAM-1 by 15% [95% CI, −17% to −13%; all P < 0.001; Table 2].

After the 24-week ramipril intervention, the change in Ang-II levels correlated inversely in the univariate analysis with the change in maximum walking time (r = −0.45; P = 0.001) and the change in femoral volume flow (r = −0.43; P = 0.001; Table 4).

The change in angiogenic markers, VEGF-A, and FGF-2 positively correlated with the change in maximum walking time (r = 0.51; P < 0.001, and r = 0.28; P = 0.001, respectively; Table 4) and the change in femoral volume flow (r = 0.27; P = 0.04, and r = 0.25; P = 0.02, respectively; Table 4). The change in D-dimer, TAT, hsCRP, OPN, sVCAM-1, and sICAM-1 levels correlated inversely in the univariate analysis with the change in maximum walking time (with r values ranging from −0.58 to −0.28; P = 0.001 for all; Table 4).

In addition, the change in D-dimer, TAT, OPN, sVCAM-1, and sICAM-1 levels correlated inversely with the change in volume flow in the common femoral artery proximal to the site of stenosis (with r values ranging from −0.51 to −0.22; P ranging from <0.001 to 0.02; Table 4). The significance of all correlations remained unchanged when age, sex, body mass index, diabetes mellitus, hypertension, cigarette smoking, medication use, and ABI were included as covariates (Table 4).

**Discussion**

The current study provides unique clinical insights regarding the actions of ramipril to improve functional capacity in the setting of a randomized controlled trial. Ramipril was associated with changes in 9 circulating biomarkers, which are linked with mechanisms including angiogenesis/arteriogenesis, inflammation, thrombosis, and leukocyte adhesion. These mechanisms are plausible candidates contributing to the increased functional capacity and the long-term cardiovascular risk reduction resulting from treatment with this ACE inhibitor in patients with PAD.1-27

The most marked changes were in FGF-2 (64% increase) and VEGF-A (38% increase), factors associated with both arteriogenesis. The reduction in D-dimer (by 24%) is also notable and only moderately lower than reported for the established antithrombotic drug warfarin, for which the reported reduction in plasma D-dimer ranges from 32% to 58%.28,29 The changes observed contribute to a rational understanding of the functional improvements associated with ramipril in patients with intermittent claudication.

**Increased Muscle Perfusion**

Ramipril-induced increase in functional capacity as assessed by maximum walking time was associated with an increase in femoral flow at a site upstream from the main stenosis, but no change
at the sites of major stenosis. This observation is consistent with an elevation in total leg perfusion mediated via collaterals between the stenotic site and the site of blood flow measurement 5 cm upstream from the major stenosis. The mechanisms increasing collateral flow are likely to be multiple and include vasodilatation, arteriogenesis, angiogenesis, and reduced thrombosis. Our data, although associative and indirect in nature, do provide support for these mechanisms and are consistent with the known mechanisms of action of ACE inhibitors.

### Table 2. Changes in Blood Parameters of the Study Population

<table>
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<tr>
<th></th>
<th>No. of Participants</th>
<th>Baseline</th>
<th>6 mo</th>
<th>Within-Group Changes</th>
<th>Between-Group Changes†</th>
<th>P Value*</th>
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<td>Creatinine, μmol/L</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>76.5 (25.8)</td>
<td>79.0 (23.2)</td>
<td>2.5 (11.0)</td>
<td>1.6 (1.4 to 4.8)</td>
<td>0.06</td>
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<tr>
<td>Ramipril</td>
<td>82</td>
<td>74.3 (22.6)</td>
<td>78.7 (21.9)</td>
<td>4.1 (9.8)</td>
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<td>Ang-II, pg/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>18.3 (2.4)</td>
<td>18.9 (2.9)</td>
<td>0.6 (1.9)</td>
<td>-5.1 (-5.6 to -4.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>Ramipril</td>
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<td>17.7 (2.4)</td>
<td>13.2 (2.5)</td>
<td>-4.5 (1.6)</td>
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<td>VEGF-A, pg/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>187 (71)</td>
<td>186 (69)</td>
<td>-1 (11)</td>
<td>64 (58 to 70)</td>
<td>&lt;0.001</td>
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<td>Ramipril</td>
<td>82</td>
<td>184 (60)</td>
<td>247 (65)</td>
<td>63 (24)</td>
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<td>FGF-2, pg/mL</td>
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<td>Placebo</td>
<td>83</td>
<td>41 (21)</td>
<td>39 (20)</td>
<td>-2 (4)</td>
<td>18 (13 to 23)</td>
<td>&lt;0.001</td>
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<tr>
<td>Ramipril</td>
<td>82</td>
<td>41 (20)</td>
<td>56 (22)</td>
<td>16 (24)</td>
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<td>D-dimer, μg/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>1.00 (0.19)</td>
<td>1.05 (0.23)</td>
<td>0.05 (0.18)</td>
<td>-0.26 (-0.32 to -0.20)</td>
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<td>Ramipril</td>
<td>82</td>
<td>1.10 (0.26)</td>
<td>0.89 (0.23)</td>
<td>-0.21 (0.18)</td>
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<td>vWF, ng/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>808 (296)</td>
<td>835 (301)</td>
<td>28 (17)</td>
<td>-304 (-317 to -291)</td>
<td>&lt;0.001</td>
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<td>82</td>
<td>910 (304)</td>
<td>634 (419)</td>
<td>-276 (67)</td>
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<td>TAT, ng/mL</td>
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<tr>
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<td>3.19 (0.97)</td>
<td>3.31 (0.97)</td>
<td>0.11 (0.19)</td>
<td>-0.57 (-0.69 to -0.45)</td>
<td>&lt;0.001</td>
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<td>3.68 (1.06)</td>
<td>3.18 (0.79)</td>
<td>-0.46 (0.52)</td>
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<td>hsCRP, mg/L</td>
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<td>2.43 (0.69)</td>
<td>2.48 (0.67)</td>
<td>0.05 (0.17)</td>
<td>-0.25 (-0.30 to -0.20)</td>
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<td>2.08 (0.79)</td>
<td>1.88 (0.77)</td>
<td>-0.20 (0.15)</td>
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<td>OPN, ng/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>73.7 (12.9)</td>
<td>73.5 (11.8)</td>
<td>-0.1 (3.2)</td>
<td>-8.6 (-9.9 to -7.2)</td>
<td>&lt;0.001</td>
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<td>Ramipril</td>
<td>82</td>
<td>74.2 (11.7)</td>
<td>65.6 (8.8)</td>
<td>-8.7 (5.5)</td>
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<td>sVCAM-1, ng/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>801 (154)</td>
<td>830 (157)</td>
<td>29 (35)</td>
<td>-131 (-209 to 53)</td>
<td>&lt;0.001</td>
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<td>Ramipril</td>
<td>82</td>
<td>788 (163)</td>
<td>697 (134)</td>
<td>-102 (13)</td>
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<td>sICAM-1, ng/mL</td>
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<tr>
<td>Placebo</td>
<td>83</td>
<td>244 (75)</td>
<td>253 (74)</td>
<td>9 (15)</td>
<td>-32 (-36 to -28)</td>
<td>&lt;0.001</td>
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<tr>
<td>Ramipril</td>
<td>82</td>
<td>248 (72)</td>
<td>226 (71)</td>
<td>-23 (11)</td>
<td></td>
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</tr>
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</table>

*Data are presented as mean (SD) unless otherwise indicated. Ang-II indicates angiotensin II; FGF-2, fibroblast growth factor-2; hsCRP, high-sensitivity C-reactive protein; OPN, osteopontin; sICAM-1, soluble intracellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TAT, thrombin-antithrombin III; VEGF-A, vascular endothelial growth factor-A; and vWF, von Willebrand factor.

*By analysis of covariance with terms for treatment and baseline values.

†Expressed as mean (95% confidence interval).

Vasodilatation

ACE inhibitor treatment can lead to vasodilatation through a reduction in Ang-II, sympathetic inhibition, but also improvement in endothelial function through the preservation of BK (Figure 2). Increased levels of BK promote the formation and release of NO, which promotes vasodilatation through well-described mechanisms (Figure 2).

### Angiogenesis/Arteriogenesis

Given that Ang-II is angiogenic in vitro and in vivo via actions on the angiotensin type 1 receptor, the assumption might be that the inhibition of Ang-II synthesis via ACE inhibitors would lead to reduced angiogenesis (Figure 2). However, studies support just the opposite, demonstrating that ACE inhibitors increase vascular density in several models including the rat limb muscle model. Furthermore, it has been
reported that treatment with an angiotensin receptor blocker was not associated with enhancement of the coronary collateral circulation, whereas treatment with an ACE inhibitor promoted coronary collateral formation in patients with coronary artery disease. These findings suggest that the differences between the effects of treatment with an ACE inhibitor and an angiotensin receptor blocker on the enhancement of collateral circulation are via BK signaling. Li et al demonstrated that ACE inhibition enhanced NO formation and tissue VEGF levels through the angiotensin type 2 receptor in the setting of tissue ischemia in angiotensin type 1 receptor–knockout mice. These effects seemed to be mediated via elevation in BK as is the case with ACE inhibitor–induced arteriogenesis and angiogenesis via its actions on the BK2 receptor. The stimulation of the BK2 receptor induces the activation of endothelial NO synthase (eNOS), and the production of NO induces the synthesis of angiogenic factors where multiple agents may be necessary. The lack of efficacy may relate to the administration of single growth factors where multiple agents may be necessary to activate endogenous pathways promoting angiogenesis.

Further studies have implicated BK as the mediator of ACE inhibitor–induced angiogenesis during ischemia via its actions on the BK2 receptor. The stimulation of the BK2 receptor induces the activation of endothelial NO synthase (eNOS), and the production of NO induces the synthesis of the angiogenic factor VEGF (Figure 2). Consistent with these mechanisms, ramipril therapy was associated with a 38% increase in the plasma levels of VEGF-A in our PAD cohort. In addition, BK acting via the BK1 receptor upregulates the arteriogenic/angiogenic factor FGF-2 via the eNOS pathway (Figure 2). The marked 64% increase in plasma FGF-2 levels associated with ramipril treatment in the current study suggests that this mechanism is an important target for ACE inhibition in the setting of intermittent claudication.

To our knowledge, this is the first randomized controlled trial to systematically examine the plasma levels of VEGF-A and FGF-2 in patients with PAD after ramipril therapy. Other previous studies in patients with either hypertension or type 1 diabetes mellitus have been equivocal, owing to issues including small sample size, short intervention duration, and absence of a placebo group.

In terms of clinical evidence for angiogenesis in association with ACE inhibition, in the context of vascular disease, ACE inhibitor treatment was associated with a progression of coronary collateral circulation in patients with coronary artery disease, likely via BK2 receptor–induced NO synthesis and thus downstream effects on VEGF. Further supporting the role of ACE inhibition in human angiogenesis, Min et al demonstrated that in patients with coronary artery disease, 1 to 3 weeks of ramipril treatment increased the levels, proliferation, migration, adhesiveness, and tube formation of cultured blood-derived endothelial progenitor cells, which play a central role in angiogenesis, possibly through an eNOS/VEGF mechanism. Similarly, in a more recent study, ramipril therapy for 20 days increased the circulating endothelial progenitor cells as measured by flow cytometry in patients with acute coronary syndrome.

In terms of peripheral ischemia, therapeutic angiogenesis has emerged as a potential strategy for the treatment of patients with PAD. In a meta-analysis of trials that included gene- and cell-based therapies in PAD, the investigators concluded that these therapies have the potential for clinical benefit. However, certain randomized clinical trials have shown that the administration of angiogenic factors such as VEGF-A and FGF-1 provided no clinical benefit in patients with debilitating PAD and critical limb ischemia, respectively. The lack of efficacy may relate to the administration of single growth factors where multiple agents may be necessary to activate endogenous pathways promoting angiogenesis.

In addition, these trials are conducted in patients with either disabling intermittent claudication or critical limb ischemia, conditions where the capacity to stimulate new blood vessel growth may be extremely limited. Further clinical trials addressing these issues are required to determine the viability of these approaches.
The elevation in BK also has implications for thrombosis, including the activation of plasminogen and the inhibition of both platelet aggregation and leukocyte adhesion to the endothelium surface. Through these mechanisms, BK prevents or reduces the occurrence of thrombosis and may inhibit haemostatis, contributing to improved tissue perfusion.

Some of the proteins involved in, stimulated by, or associated with thrombosis include fibrinogen, D-dimer, vWF, and TAT complex (Figure 2). The significant reduction

Table 4. Univariate Correlations With % ∆Maximum Walking Time and % ∆Volume Flow in Patients Who Received Ramipril Therapy

<table>
<thead>
<tr>
<th></th>
<th>r Value</th>
<th>P Value</th>
<th>r Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ∆Maximum walking time, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% ∆Ang-II, pg/mL</td>
<td>-0.45</td>
<td>0.001</td>
<td>-0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆VEGF-A, pg/mL</td>
<td>0.51</td>
<td>&lt;0.001</td>
<td>0.47</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆FGF-2, pg/mL</td>
<td>0.28</td>
<td>0.001</td>
<td>0.24</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆D-dimer, μg/mL</td>
<td>-0.37</td>
<td>0.001</td>
<td>-0.35</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆WF, ng/mL</td>
<td>-0.11</td>
<td>0.17</td>
<td>-0.12</td>
<td>0.18</td>
</tr>
<tr>
<td>% ∆TAT, ng/mL</td>
<td>-0.31</td>
<td>0.001</td>
<td>-0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>% ∆hsCRP, μg/L</td>
<td>-0.28</td>
<td>0.001</td>
<td>-0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>% ∆OPN, ng/mL</td>
<td>-0.49</td>
<td>0.001</td>
<td>-0.48</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆sVCAM-1, ng/mL</td>
<td>-0.44</td>
<td>0.001</td>
<td>-0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆sICAM-1, ng/mL</td>
<td>-0.58</td>
<td>0.001</td>
<td>-0.46</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆Volume flow, limiting leg ABI, mL/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% ∆Ang-II, pg/mL</td>
<td>-0.43</td>
<td>0.001</td>
<td>-0.36</td>
<td>0.01</td>
</tr>
<tr>
<td>% ∆VEGF-A, pg/mL</td>
<td>0.27</td>
<td>0.04</td>
<td>0.23</td>
<td>0.05</td>
</tr>
<tr>
<td>% ∆FGF-2, pg/mL</td>
<td>0.25</td>
<td>0.02</td>
<td>0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>% ∆D-dimer, μg/mL</td>
<td>-0.22</td>
<td>0.02</td>
<td>-0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>% ∆WF, ng/mL</td>
<td>-0.10</td>
<td>0.29</td>
<td>-0.09</td>
<td>0.35</td>
</tr>
<tr>
<td>% ∆TAT, mg/L</td>
<td>-0.30</td>
<td>0.02</td>
<td>-0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>% ∆hsCRP, μg/L</td>
<td>-0.01</td>
<td>0.36</td>
<td>0.01</td>
<td>0.89</td>
</tr>
<tr>
<td>% ∆OPN, ng/mL</td>
<td>-0.51</td>
<td>&lt;0.001</td>
<td>-0.39</td>
<td>0.001</td>
</tr>
<tr>
<td>% ∆sVCAM-1, ng/mL</td>
<td>-0.25</td>
<td>0.02</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>% ∆sICAM-1, ng/mL</td>
<td>-0.49</td>
<td>0.001</td>
<td>-0.40</td>
<td>0.001</td>
</tr>
</tbody>
</table>

ABI indicates ankle brachial index; Ang-II, angiotensin II; FGF-2, fibroblast growth factor-2; hsCRP, high-sensitivity C-reactive protein; OPN, osteopontin; sICAM-1, soluble intracellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TAT, thrombin-antithrombin III; VEGF-A, vascular endothelial growth factor-A; and vWF, von Willebrand factor.

*Adjusted for age, sex, body mass index, diabetes mellitus, hypertension, cigarette smoking, medication use, and ABI as covariates.

Thrombosis

The elevation in BK also has implications for thrombosis, including the activation of plasminogen and the inhibition of both platelet aggregation and leukocyte adhesion to the endothelium surface. Through these mechanisms, BK prevents or reduces the occurrence of thrombosis and may inhibit haemostatis, contributing to improved tissue perfusion. Some of the proteins involved in, stimulated by, or associated with thrombosis include fibrinogen, D-dimer, vWF, and TAT complex (Figure 2). The significant reduction

Figure 2. Potential mechanisms by which ramipril increases lower limb perfusion. Increased blood flow may be mediated via (1) angiogenesis/arteriogenesis through both the renin–angiotensin system (via AT2R) and the bradykinin system, (2) reduction in thrombosis via NO mechanisms mediated by the preservation of bradykinin, and (3) vasodilatation through reduction in angiotensin II. Parameters measured in the current study are shown in red. AT1R indicates angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; BK1R, bradykinin 1 receptor; BK2R, bradykinin 2 receptor; cGMP, cyclic guanosine monophosphate; eNOS, endothelial NO synthase; FGF-2, fibroblast growth factor-2; NO, nitric oxide; sGC, soluble guanylyl cyclase; SNS, sympathetic nervous system; TAT, thrombin-antithrombin III; VEGF-A, vascular endothelial growth factor-A; and vWF, von Willebrand factor.
in plasma D-dimer, vWF, and TAT levels associated with ramipril therapy further supports the antiatherothrombotic action of ACE inhibitors, which are likely mediated via the BK pathway. ACE inhibition reduces these plasma thrombotic markers to an extent comparable with conventional anticoagulant therapies, such as warfarin and aspirin. It should be noted that although factors affecting coagulation and blood rheology do play a role in intermittent claudication via the impact on microvascular blood flow, there is little evidence that current anticoagulant therapies improve walking ability. The current use of antiplatelet agents either alone or in combination with warfarin is the subject of ongoing research and may reveal specific PAD subgroups benefiting from such therapy.

Interestingly, vWF has also been demonstrated to be a negative modulator of angiogenesis. The molecular basis of this vWF-dependent effect is still not completely clear; however, experimental data point to vWF as a negative regulator of VEGF-dependent angiogenesis via multiple intracellular and extracellular pathways involving VEGFR, extracellular matrix proteins such as αvβ3 integrin, and angiopoietin-2 (Figure 2).

Skeletal Muscle Glucose Uptake
In combination with mechanisms that may enhance blood flow, ACE inhibition may improve walking ability by enhancing glucose uptake and metabolism in skeletal muscle. This effect has been attributed to BK and downstream effects on NO, which increases the delivery of both insulin and glucose to muscle. Finally, BK also directly stimulates insulin-dependent and insulin-independent glucose uptake into muscle.

Inflammation and Adhesion
In addition to the mechanisms that may improve walking ability via effects on leg blood flow and glucose uptake, ramipril therapy was also associated with a reduction in markers of inflammation and leukocyte adhesion, which may reduce longer-term atherosclerotic risk. Ang-II functions as a potent inflammatory stimulus and upregulates the expression of many redox-sensitive cytokines, chemokines, and growth factors involved in the pathogenesis of atherosclerosis, including CRP and OPN (Figure 3). CRP has been shown to induce leukocyte adhesion molecule expression in endothelial cells, suggesting a local proinflammatory function.

Ramipril therapy was associated with a 13% reduction in plasma hsCRP levels in our PAD cohort. In agreement with our findings, ACE inhibition has previously been shown to reduce hsCRP levels by ≈46% in patients with impaired cardiac function.

The effects of ACE inhibitor therapy on CRP and cardiovascular outcome are controversial. In the Prevention of Events With Angiotensin-Converting Enzyme Inhibition (PEACE) trial, there were no significant interactions between hsCRP levels and effects of trandolapril on adverse cardiovascular events. However, the PEACE trial included patients with stable coronary artery disease with baseline hsCRP levels that were significantly lower compared with those in our PAD cohort; this may account for the lack of effect of trandolapril reported in PEACE.

However, several studies have demonstrated a correlation between CRP reduction and improved outcome. In Di Napoli and Papa, concomitant therapy with an ACE inhibitor at the time of an acute stroke was associated with lower CRP plasma concentration and reduced 2-year cardiovascular risk. In other therapeutic contexts, statin therapy has also been associated with reductions in hsCRP ranging from 12% to 37% and associated reduction in the incidence of major cardiovascular events.

OPN can function as a chemotactic cytokine, regulating immune cell function and promoting the adhesion, migration, and activation of macrophages. Several animal studies
have shown that Ang-II upregulates OPN expression, whereas the blockade of Ang-II by using an ACE inhibitor, such as ramipril, suppresses inflammatory cytokine OPN expression.53 Our data demonstrate that ramipril was associated with a modest 12% reduction in plasma OPN levels.

Furthermore, Ang-II also modulates vascular inflammation by regulating the expression of adhesion molecules, such as ICAM-1, ICAM-2, and VCAM-1, leading to leukocyte activation and recruitment7 (Figure 3). In our PAD cohort, ACE inhibition therapy resulted in a 14% reduction in sVCAM-1 and 15% reduction in sICAM-1. The increased formation of NO through the BK2 receptor can, via reduction in vWF, also reduce the adhesion of leukocytes to the vascular endothelium76 and inhibit platelet aggregation. vWF is also tightly associated with the inflammatory response. For example, reduced levels of vWF cause reductions in leukocyte recruitment77 (Figure 3).

It is thus clear that the well-documented reduction in Ang-II levels and increase in BK levels after ACE inhibition80,31,78 would mediate a variety of anti-inflammatory and antiadhesion actions, which could reduce long-term atherothrombotic risk.

Strengths and Limitations

The effects of ramipril were examined in the context of a robust randomized, placebo-controlled study design within a well-defined clinical population. Although the absolute concentration of all measured biomarkers may vary between tissues and blood, there is evidence of significant correlations between concentrations in these different compartments.17-23,79-82 VEGF plasma concentration has been shown to vary approximately linearly with VEGF secretion rate in a biophysically accurate compartment model.83 This model permits study the distribution of VEGF isoforms, including 165 and 121, in both tissue (matrix-bound, cell surface receptor–bound, and free VEGF isoforms) and blood.17 Such models have also demonstrated that VEGF concentration in blood, but not in tissue, is dependent on the vascular permeability of healthy tissue.17,23,83

The net arteriogenic/angiogenic effects of measured VEGF isoforms (165 and 121) would also be influenced by other VEGF isoforms and receptors not assessed in this study. These include the VEGF_A6 isoforms, generated through alternative splicing, which are generally antiangiogenic.84,85 The soluble VEGF receptors were also not measured. Based on mouse models, the soluble VEGFR, would have been of particular interest because its overexpression in PAD may account for blunted angiogenic response.86

The reduction in plasma Ang-II demonstrates that ramipril elicited effective ACE inhibition. Although it was not possible to measure plasma BK levels in our cohort, there is substantial evidence in the literature showing elevation in circulating BK concentration after ACE inhibition.57,88 Furthermore, the evaluation of walking times and femoral blood flow permits the interpretation of changes in circulating factors from a functional perspective. It must be acknowledged, however, that the relationships between changes in functional and circulating parameters are associative, and that cause and effect cannot be directly established. In addition, because femoral blood flow was only assessed at rest, we cannot determine whether ramipril increased blood flow during exercise. Finally, it is possible that our observations resulted from a combination of effects of ramipril leading to increased physical activity. However, because physical activity was not assessed in the current cohort, it is not possible to determine whether this was the case.

Conclusions

Ramipril therapy was associated with an increase in markers of angiogenesis/arteriogenesis and a reduction in the markers of thrombosis, inflammation, and leukocyte adhesion, suggesting that multiple interrelated mechanisms are likely to contribute to the beneficial effects of ramipril in patients with PAD. Collectively, we observed positive correlations between the change in maximum walking time and change in angiogenic/arteriogenic markers, and negative correlations with markers of adhesion, thrombosis, and inflammation. This study not only informs potential mechanisms by which ramipril mediates improved functional capacity but also provides guidance for future therapeutic strategies directed to improving functional capacity in patients with PAD with intermittent claudication. Specfically, the most marked changes were in VEGF-A, FGF-2, and D-dimer, suggesting that approaches targeting these mechanisms could be targets for intervention in future randomized clinical trials. New strategies are needed to improve mobility and reduce disability among individuals with PAD89 in the context of the current shift in global disease burden to chronic disease and more years lived with disability.

Sources of Funding

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Disclosures

None.

References


### What Is Known?

- In patients with peripheral artery disease (PAD) with intermittent claudication, treatment with an angiotensin-converting enzyme (ACE) inhibitor, ramipril, increases maximum walking time.
- Treatment of patients with PAD with ramipril also improves quality of life.
- Leg blood flow changes suggest that ramipril increases collateral blood flow; however, the mechanisms contributing to this effect are unknown.

### What New Information Does This Article Contribute?

- In patients with PAD with intermittent claudication, ramipril induces changes in circulating biomarkers, which include increases in markers of angiogenesis/arteriogenesis and reductions in markers of inflammation, thrombosis, and leukocyte adhesion.
- Treatment with ramipril increased circulating angiogenic/arteriogenic markers, suggesting that it may induce the growth of new blood vessels that contribute to improved leg perfusion and walking ability.
- Whether the changes in circulating biomarkers are primary effects of ACE inhibition or secondary to vasodilatation and possibly increased physical activity requires further investigation.

Novelty and Significance

PAD affects 200 million people worldwide and is currently under-diagnosed and undertreated, despite the high mortality rate associated with this condition. Approximately one third of patients with PAD experience intermittent claudication, which substantially limits mobility and quality of life. Ramipril has recently been shown to dramatically improve walking ability and quality of life in patients with PAD with intermittent claudication. However, its mechanisms of action remain unclear. Although vasodilatation is an expected mechanism associated with ACE inhibition, the current study implicates angiogenesis/arteriogenesis and reductions in inflammatory, thrombotic, and leukocyte adhesion processes among the additional actions of ramipril, which may contribute to improved leg perfusion and walking ability. Of these, the largest effects were with respect to increases in circulating angiogenic/arteriogenic markers, suggesting that ramipril may contribute to the growth of new blood vessels; however, this effect of ramipril treatment requires further substantiation in future animal and clinical studies.
Potential Vascular Mechanisms of Ramipril Induced Increases in Walking Ability in Patients With Intermittent Claudication

Anna A. Ahimastos, Celine Latouche, Alaina K. Natoli, Medini Reddy-luthmoodoo, Jonathan Golledge and Bronwyn A. Kingwell

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The authors of the following article, which published in the March 28, 2014 issue of *Circulation Research*, have requested that it be retracted from publication:


A recent internal sub-analysis by Baker IDI Heart and Diabetes Institute (“the Institute”) of the data presented in this article revealed anomalies that triggered an investigation, which resulted in an admission of fabricated results by Dr Anna Ahimastos. Dr Ahimastos is both the first and corresponding author of this article, and was responsible for data collection and integrity. According to the Institute’s finding, no other co-authors were involved in this misrepresentation. In addition, no data from the Townsville and Brisbane recruitment centers were included in this publication, and data from those centers remains valid. All authors recognize the seriousness of this issue and apologize unreservedly to the editors, reviewers, and readers of *Circulation Research*. Although a system of Good Clinical Practice was in place, the Institute vows to review and strengthen Clinical Governance and audit procedures to minimize the chance of possible recurrence of such behavior.

In accordance with the policies of the Baker IDI Heart and Diabetes Institute, the authors have requested to retract this article.