Ramipril Reduces Large-Artery Stiffness in Peripheral Arterial Disease and Promotes Elastogenic Remodeling in Cell Culture

Anna A. Ahimastos, Alaina K. Natoli, Adam Lawler, Peter A. Blombery, Bronwyn A. Kingwell

Abstract—Ramipril improves cardiovascular outcome in patients with peripheral arterial disease; however, the precise mechanisms of benefit remain to be elucidated. The effect of ramipril on large-artery stiffness in patients with peripheral arterial disease was examined. In addition, we determined the effect of ramipril on extracellular matrix from human aortic smooth muscle cell culture. Forty patients with peripheral arterial disease were randomized to receive ramipril, 10 mg once daily or placebo for 24 weeks. Arterial stiffness was assessed globally via systemic arterial compliance and augmentation index (carotid tonometry and Doppler velocimetry), and regionally via carotid–femoral pulse wave velocity. Angiotensin-converting enzyme inhibition increased arterial compliance by 0.10 ± 0.02 mL/mm Hg, \( P < 0.001 \), all probability values relative to placebo) and reduced pulse wave velocity by 1.7 ± 0.2 m/s \( P < 0.001 \), augmentation index by 4.1 ± 0.3\% \( P < 0.001 \), and systolic blood pressure by 5 ± 1 mm Hg \( P < 0.001 \). Ramipril did not reduce mean arterial pressure significantly compared with placebo \( P = 0.59 \). In cell culture, ramipril decreased collagen deposition by >50\% and increased elastin and fibrillin-1 deposition by >3- and 4-fold respectively (histochemistry and immunohistochemistry). Fibrillin-1 gene expression was increased 5-fold (real-time reverse-transcriptase polymerase chain reaction). Ramipril also reduced gene and protein (Western) expression of both matrix metalloproteinase (MMP)-2 and MMP-3. In conclusion, ramipril promoted an elastogenic matrix profile that may contribute to the observed clinical reduction in large-artery stiffness and carotid pressure augmentation, which occurred independently of mean arterial blood pressure reduction in patients with peripheral arterial disease. (Hypertension. 2005;45:1194-1199.)

Key Words: angiotensin-converting enzyme ■ arteries ■ extracellular matrix

E levated arterial stiffness, is emerging as an important predictor of cardiovascular outcome in the healthy elderly \(^1\) and in a variety of disease populations \(^2\) \-\(^4\) Furthermore, reducing arterial stiffness may have prognostic benefit \(^5\), making it a key target for therapy. Carotid and femoral arterial stiffness is elevated in patients with peripheral arterial disease (PAD) \(^6\) \-\(^7\) however, no previous study has examined the efficacy of therapeutic interventions to reverse such effects. Angiotensin-converting enzyme (ACE) inhibitors are well-established to protect against coronary events and in the Heart Outcomes Prevention Evaluation (HOPE) trial, the ACE inhibitor ramipril reduced cardiovascular endpoints compared with placebo in patients with established atherosclerotic disease, including PAD \(^8\). This effect was independent of blood pressure changes and may have been caused by structural effects of ramipril on the vasculature.

Generally, antihypertensive drugs reduce arterial stiffness passively through mean arterial pressure reduction. Some classes, particularly ACE inhibitors, may have additional effects on arterial wall structure contributing to a reduction in arterial stiffness \(^9\) \-\(^10\). ACE inhibitors have been shown to increase the elastin to collagen ratio in a number of disease models \(^11\) \-\(^13\). Though of great interest, the relevance of these animal models to humans is unknown. Furthermore, these chronic studies cannot specifically examine the direct effects of ACE inhibitors on arterial matrix protein deposition independently of their hemodynamic actions.

We examined the effects of ramipril treatment for 24 weeks on large-artery stiffness and pressure wave reflection in patients with PAD. In addition, we examined the effect of ramipril on matrix protein deposition and gene and protein expression of specific matrix metalloproteinases (MMPs) in cultured human aortic smooth muscle cells. We hypothesized that ramipril would reduce large-artery stiffness and carotid pressure augmentation in these patients and would increase the elastin to collagen ratio in cell culture.

Methods

Study Design

Clinical

Forty patients (mean age ± SD; 66 ± 4 years) with PAD gave written informed consent to participate in the project, which was approved...
by the Ethics Committee of the Alfred Hospital and performed in accordance with the Declaration of Helsinki (2000). All participating patients had an ankle–brachial index (ABI) of $<0.9$ at rest in at least one leg, history of intermittent claudication (unilateral or bilateral) that was stable for 6 months, evidence of superficial femoral artery stenosis or occlusion on duplex scan, blood pressure $\geq 160/90$ mm Hg on screening, and a stable medication regimen for at least 6 months. Patients treated for hypertension, with limiting coronary artery disease, renal failure, type 2 diabetes mellitus, or proximal disease at or above the level of the inguinal ligament, were excluded from the study. A randomized, double-blind, parallel group study was conducted comparing the effects of 24 weeks of treatment with ramipril (10 mg, once daily, n = 20; Aventis Pharma Pty Ltd) versus placebo (n = 20) on large-artery stiffness. Participants were requested to refrain from exercise, smoking, and caffeine for 24 hours before the experimental trial. On the morning of testing, patients rested in a supine position for 15 minutes. Blood pressure and arterial stiffness were assessed before and after both interventions.

Cell Culture
Primary human aortic smooth muscle cells were incubated for 10 weeks with clinically relevant concentrations of the active metabolite of ramipril, ramiprilat. At completion of the 10-week period, deposition of elastin and collagen was examined by histochemistry and deposition of fibrillin-1 by immunohistochemistry. Gene expression of fibrillin-1, MMP-2, and MMP-3 were measured using real-time reverse-transcriptase polymerase chain reaction (RT-PCR), whereas protein levels were determined by Western blotting.

Resting Blood Pressure
Supine resting brachial arterial blood pressure and heart rate were measured 3 times, at 3-minute intervals, using an automated oscillometric blood pressure monitor (Dinamap Vital Signs Monitor 18465X; Criticon), after 15 minutes of quiet rest in a temperature-controlled room.

Ankle/Brachial Index
Ankle systolic blood pressure was measured in the posterior tibial and dorsalis pedis arteries with a nondirectional Doppler flow detector (Parks Medical Electronics Inc), a pencil probe (9.3 MHz), and a standard size ankle blood pressure cuff. Brachial blood pressure was measured using a manual oscillometric sphygmomanometer (PyMaH Corp). ABI was calculated as the ratio of the highest systolic blood pressure in each ankle from the right and left posterior tibial or dorsalis pedis arteries, divided by the highest brachial systolic blood pressure.

Arterial Stiffness
Arterial stiffness was assessed globally via systemic arterial compliance (carotid tonometry, and Doppler velocimetry) and augmentation index (carotid tonometry), and regionally via central pulse wave velocity. Systemic arterial compliance was determined noninvasively using calculations based on the area method of Liu et al$^{14}$ and a 2-element Windkessel model of the arterial system as described previously.$^{16}$ This method has been validated to primarily assess the properties of the large vessels including the aorta and carotid arteries.$^{16}$ Carotid augmentation index was calculated as the difference between the pressure at the first and second systolic peaks of the central arterial waveform, expressed as a percentage of the pulse pressure.$^{17}$ Pulse wave velocity (PWV) is related directly to aortic stiffness (excluding the most proximal region$^{16}$) and was measured centrally between the right carotid and femoral arteries, by simultaneous applanation tonometry (SPT-301; Miller Instruments).$^{18}$

Cell Culture
Human aortic smooth muscle cells from an 11-month-old white female (CRL-1999; American Type Culture Collection) were cultured in Dulbecco’s Modified Eagle Medium/Hams F12K 50:50, phenol red-free (Invitrogen) containing 10% fetal calf serum, 90 mmol/L NaHCO$_3$, 4 mmol/L L-glutamine, 100 U penicillin, 100 μg streptomycin at 37°C, and 5% CO$_2$. Culture medium was changed every 3 days. Cells were cultured with ramiprilat for 10 weeks (Aventis Pharma Deutschland) at a concentration calculated to correspond to clinical plasma concentrations achieved with the doses of ramipril used in the human trial (35 ng/mL, n = 6 per protein).

Matrix Deposition (Collagen, Elastin, Fibrillin-1)
Deposition of collagen types, I, II, and III was assessed using picrosirius red histochemistry and elastin by orcein histochemistry. Deposition of fibrillin-1 was examined through immunohistochemistry. Normal horse serum (CSL Biosciences) was used to block nonspecific binding. A mouse anti-human fibrillin-1 monoclonal antibody (Clone 11C1.3; Neomarkers) was used in conjunction with a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories).

Light microscope images (Olympus Microscope BX-50; Olympus Corporation) were captured at a magnification of $\times$850 with a video camera (JVC Color Camera KY-F35BE; JVC) coupled to live imaging software (Optimas Imaging, Version 6.51; Optimas Inc.). Using a color threshold detection system, which was standardized for each protein, the proportion of staining within each field was calculated as a percentage of the total field. Twenty fields per plate were analyzed and averaged.

Real-Time RT-PCR (MMP-2, MMP-3, Fibrillin-1)
MMP-2, MMP-3, and fibrillin-1 mRNA levels were determined using real-time RT-PCR sequence detection (ABI Prism 7700; Applied Biosystems). Real-time RT-PCR amplifications were prepared in duplicate on 2 separate occasions (ie, 4 times). mRNA levels were normalized to 18S rRNA (Applied Biosystems) and fold expression determined as previously described.$^{19}$

Protein Levels (MMP-2, MMP-3)
Total protein expression of MMP-2 and MMP-3 were determined by Western blotting using relevant antibodies (primary: anti-MMP-2 and anti-MMP-3, Sigma; secondary: anti-rabbit polyclonal MMP-2 and anti-mouse monoclonal MMP-3, Amersham Pharmacia Biotech), and proteins were visualized using the ECL technique. Bands/protein levels were then quantified on digitized films as the product of band density and area using Optimas 6.1 Software (Media Cybernetics, LP) and expressed relative to a control level of 1.

Statistical Analyses
The change in all clinical parameters from baseline to 24 weeks was compared between the placebo and ramipril groups using unpaired t tests. Differences in all parameters between the placebo and ramipril treatment in the cell culture studies were also assessed using unpaired t tests. All statistical analysis was performed using SPSS (Version 12.0). $P<0.05$ was deemed to be significant.

Results
Clinical
Although no patient had a previous history of hypertension before screening or had been previously treated for that condition, 17 patients had systolic blood pressure $>140$ mm Hg or diastolic pressure $>90$ mm Hg on the day of screening. Seven patients had total cholesterol levels $>5.5$ mmol/L. There was no difference in any baseline parameter including severity of PAD as evidenced by ABI for patients in the placebo and ramipril groups (Table 1). Five patients were using warfarin and 11 were treated with hypolipidemic agents.

Blood Pressure and Arterial Stiffness
Ramipril significantly reduced brachial (placebo, $-0.5\pm0.4$ mm Hg; ramipril, $-5.4\pm0.7$ mm Hg; $P<0.001$) and
This article was retracted in January 2016

### Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo Group</th>
<th>Ramipril Group</th>
<th>P</th>
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<tr>
<td>Age, y</td>
<td>67±3</td>
<td>65±6</td>
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<tr>
<td>BMI, kg/m²</td>
<td>24.4±0.6</td>
<td>24.3±0.7</td>
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<td>Brachial SBP, mm Hg</td>
<td>140±1</td>
<td>139±1</td>
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<td>Brachial DBP, mm Hg</td>
<td>89±2</td>
<td>85±2</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.04±0.14</td>
<td>5.08±0.13</td>
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<td>LDL cholesterol, mmol/L</td>
<td>2.82±0.15</td>
<td>2.80±0.14</td>
<td>0.91</td>
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<td>HDL cholesterol, mmol/L</td>
<td>1.37±0.06</td>
<td>1.37±0.09</td>
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<td>Triglycerides, mmol/L</td>
<td>1.70±0.19</td>
<td>1.58±0.18</td>
<td>0.66</td>
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<tr>
<td>Resting ABI, left</td>
<td>0.63±0.03</td>
<td>0.69±0.03</td>
<td>0.18</td>
</tr>
<tr>
<td>Resting ABI, right</td>
<td>0.70±0.04</td>
<td>0.73±0.03</td>
<td>0.59</td>
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<tr>
<td>SAC, mL/mm Hg</td>
<td>0.19±0.02</td>
<td>0.16±0.01</td>
<td>0.35</td>
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<tr>
<td>PWVc, m/s</td>
<td>9.8±0.2</td>
<td>9.5±0.3</td>
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<td>AI, %</td>
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<tr>
<td>Ts, ms</td>
<td>126±4</td>
<td>120±4</td>
<td>0.22</td>
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</table>

Abbreviations: ABI indicates ankle brachial index; AI, augmentation index; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWVc, carotid pulse wave velocity central; SAC, systemic arterial compliance; SBP, systolic blood pressure; Ts, time to first systolic inflection point.

All parameters are mean±SEM except age, which is mean; SD.

Central systolic blood pressure (placebo, −0.3±0.3 mm Hg; ramipril, −9±1 mm Hg; P<0.001, Figure 1, upper panels). Diastolic blood pressure was also reduced (placebo, −0.7±0.8 mm Hg; ramipril, −6.3±1.4 mm Hg; P=0.002); however, the change in mean arterial pressure was not different between groups (placebo, −1.3±0.9 mm Hg; ramipril, −2.3±1.8 mm Hg; P=0.59). Brachial pulse pressure was unaffected by the ramipril intervention (Table 2); however there was a trend for reduction in central pulse pressure (Table 2). There was no difference in heart rate response between the placebo and ramipril interventions (placebo, −0.5±0.8 bpm; ramipril, 0.3±1.0 bpm; P=0.53).

Ramipril increased systemic arterial compliance by 0.10±0.02 mL/mm Hg, and this was significantly different from placebo (−0.004±0.003 mL/mm Hg; P<0.001; Figure 1, lower left panel). Consistent with these findings, ramipril reduced central pulse wave velocity by 1.7±0.2 m/s (compared with 0.4±0.3 m/s for placebo; P<0.001; Figure 1, lower right panel). In addition, ramipril reduced carotid augmentation index (P<0.001; Table 2). The slower time to the first systolic inflection point after ramipril treatment (Table 2) suggests that this was largely a result of reduced pulse wave velocity. Ramipril also increased resting ABI (Table 2); however, this was caused by the reduction in brachial systolic blood pressure rather than an increase in ankle systolic pressure (Table 2). The reduction in brachial systolic pressure is likely to have resulted from both the reduction in large artery stiffness and alterations in peripheral wave reflection.

**Cell Culture**

**Matrix Deposition (Collagen, Elastin, Fibrillin-1)**

Under control conditions, collagen staining accounted for 56±4% of the visual field (Figure 2). Treatment with ramipril resulted in a reduction to 28±5% (P=0.01). Under control conditions, positive elastin staining was present in 8.3±0.1% of the visual field (Figure 2). This was significantly increased to 30±1% (P=0.001) with ramipril treatment. Ramipril thus increased the elastin/collagen ratio by >7-fold. Fibrillin-1 staining was increased from 13.0±0.3% under control conditions (Figure 2) to 64±5% (P=0.003) after treatment with ramipril. Ramipril treatment increased fibrillin-1 gene expression by >5-fold (control, 1.1±0.1; ramipril, 5.7±0.9; P<0.001).

**MMP-2 and MMP-3**

Ramipril reduced MMP-3 protein by 4-fold and mRNA by 3-fold (P<0.001; Figure 3, left panels). Similarly, ramipril reduced MMP-2 protein by 2.5-fold and reduced mRNA to negligible levels (P<0.001; Figure 3, right panels).

**Discussion**

This study demonstrates for the first time to our knowledge that treating PAD patients for 24 weeks with the ACE inhibitor ramipril reduces large-artery stiffness and carotid pressure augmentation independently of changes in mean arterial blood pressure. The mechanism underlying the reduction in cardiovascular events in PAD patients after ramipril therapy in the HOPE trial may thus include reduction in large-artery stiffness and central wave reflection. The cell culture data indicate that the reduction in arterial stiffness is likely mediated, at least in part, by an increase in large-artery extracellular matrix elastin/collagen ratio.

The reduction in arterial stiffness was accompanied by a reduction in both carotid and brachial systolic blood pressure and a trend for carotid pulse pressure reduction, which just
failed to reach statistical significance (*P* = 0.07). Brachial pulse pressure was not, however, affected by the ramipril intervention. This is likely because of the effect of ramipril on wave reflection patterns in this region. Because central pressures are more relevant to cardiac function, the difference in central and peripheral pulse pressure highlights the importance of measuring central rather than brachial blood pressure.

A large number of previous studies have examined the effect of ACE inhibitors on large-artery stiffness in both human hypertension and animal models. ACE inhibition reduces large-artery stiffness in rats with myocardial infarction and in spontaneously hypertensive rats. In humans, as little as 1 month of ACE inhibition reduced arterial wave reflection and aortic stiffness in hypertensive patients. Such effects are sustained with longer-term therapy. The current study demonstrates that similar PWV reductions are possible after 6 months ramipril therapy in PAD patients, a group with established atherosclerosis. Furthermore, the reduction in arterial stiffness and slower PWV contributed to reduced central pressure augmentation and greater resting ABI. The latter effect was caused by reduction in brachial systolic blood pressure rather than an increase in ankle systolic pressure. An increase in ABI has previously been assumed to indicate either a reduction in the severity of proximal occlusive disease in the leg or an increase in collateral blood flow around a diseased area. This study presents a further explanation for the phenomenon of a reduction in brachial systolic pressure compared with ankle systolic pressure caused by reduced arterial stiffness and consequent changes in peripheral wave reflection. This has important implications in the interpretation of drug effects on ABI in so far as an increase in ABI does not necessarily indicate an improvement in peripheral perfusion.

### Mechanisms

ACE inhibition could reduce arterial stiffness by functional mechanisms including mean pressure reduction and alteration in extracellular matrix loading via vasodilation, as well as through structural effects. Mean arterial pressure reduction was not a contributing factor in the current study. It is, however, possible that passive mechanisms linked to vasodilation contribute to reduction in arterial stiffness and carotid pressure augmentation. Such a mechanism could involve reduction in circulating angiotensin II and its downstream mediators, as well as through nitric oxide as a result of bradykinin preservation.

The persistence of these effects even after withdrawal of therapy, however, suggests that ACE inhibitors promote structural remodeling of the arterial wall. ACE inhibitors are known to decrease the development of atherosclerotic lesions through antiproliferative and antimigratory effects. In addition, these agents could also cause vascular remodeling by affecting the arterial elastin–collagen ratio. In the arterial wall, collagens play an important structural and load-bearing role.
role, whereas matrix proteins such as elastin and fibrillin-1 are essential in conveying elasticity.\textsuperscript{35} Angiotensin II stimulates both myocardial and vascular collagen expression,\textsuperscript{36} and previous studies have demonstrated that interruption of the renin-angiotensin system decreases collagen deposition\textsuperscript{37,38} and/or increases the elastin/collagen ratio.\textsuperscript{11–13,38,39} These previous in vivo animal ACE inhibition studies have not, however, examined such effects independently of changes in hemodynamics. The current cell culture data indicate that ramipril increased deposition of elastic components of the extracellular matrix, including fibrillin-1 and elastin, while reducing collagen deposition. The effect on fibrillin-1 was mediated at least in part through transcriptional upregulation. The aggregate result of these changes was an increase in the elastin to collagen ratio by \( \frac{7}{1022} \)-fold after ramipril treatment.

MMPs play an important role in vascular remodeling and can collectively degrade all components of the extracellular matrix. We focused on 2 MMPs (2 and 3) with broad substrate specificities and that are constitutively expressed in healthy vascular tissue. MMP-2 degrades elastin, fibronectin, and type IV collagen, and has been shown to increase collagen deposition through the transforming growth factor-\( \beta \) during aging.\textsuperscript{40} MMP-3 acts on fibronectin, proteoglycans, and fibrillin-1.\textsuperscript{41} Treatment with ramiprilat significantly reduced MMP-2 and MMP-3 protein. Interestingly, the concomitant reduction in mRNA suggests that ramiprilat mediated these effects through transcriptional inhibition. Although MMP activity was not examined, it would be expected to correlate with protein levels.\textsuperscript{42} ACE inhibition reduces MMP activity in animal models of hypertension\textsuperscript{44} and diabetes mellitus.\textsuperscript{42} Collectively, these data suggest that ACE inhibition decreases MMP gene expression, protein levels, and activity. Such effects likely contribute to a more elastogenic matrix profile through inhibition of atherosclerotic remodeling, reduced degradation of elastic components of the extracellular matrix (elastin and fibrillin-1), and decreased collagen deposition.\textsuperscript{40}

**Conclusion**

Ramipril reduced large-artery stiffness and carotid pressure augmentation independently of mean arterial blood pressure reduction in patients with PAD. Furthermore, in cell culture, ramipril promoted an elastogenic matrix profile, which may contribute to the observed effect on arterial stiffness. This may be an important mechanism of therapeutic benefit in this large, high-risk population.

**Acknowledgments**

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**References**


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The article, “Ramipril reduces large-artery stiffness in peripheral arterial disease and promotes elastogenic remodeling in cell culture” by Ahimastos et al (Ahimastos AA, Natoli AK, Lawler A, Blombery PA, Kingwell BA) has been retracted. The article was published in *Hypertension* (2005;45:1194–1199. doi: 10.1161/01.HYP.0000168945.44069.aa) online on May 16, 2005 and in print in the June 2005 issue of the journal.

The authors wish to retract the above article based on inadequate validation of primary data sources and data misrepresentation. An independent review of the study data was conducted following an admission of scientific misconduct by the first author, Dr Ahimastos, related to another study in peripheral arterial disease (Ahimastos, *JAMA*, 309(5):453–460, 2013). This resulted in a decision by the authors to retract the article, although the retraction has not been signed by Dr Ahimastos, who maintains the integrity of the data and validity of results. The other authors were not involved in any misconduct and apologize unreservedly to the editors, reviewers, and readers of *Hypertension*. Given the current indications for ramipril, we do not believe that there has been any negative clinical impact on patients.