Advanced glycation endproduct crosslink breaker (alagebrium) improves endothelial function in patients with isolated systolic hypertension

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Objectives **Arterial stiffening and endothelial dysfunction are hallmarks of aging, and advanced glycation endproducts (AGE) may contribute to these changes. We tested the hypothesis that AGE crosslink breakers enhance endothelial flow-mediated dilation (FMD) in humans and examined the potential mechanisms for this effect.**

Methods **Thirteen adults (nine men, aged 65 ± 2 years) with isolated systolic hypertension (systolic blood pressure >140 mmHg, diastolic blood pressure <90 mmHg or pulse pressure >60 mmHg) on stable antihypertensive therapy were studied. Subjects received placebo (2 weeks) then oral alagebrium (ALT-711; 210 mg twice a day for 8 weeks). Subjects and data analyses were blinded to treatment. Arterial stiffness was assessed by carotid augmentation index (AI) and brachial artery distensibility (ArtD) using applanation tonometry and Doppler echo, and endothelial function by brachial FMD. Serum markers of collagen metabolism and vascular inflammation were assessed.**

Results **Alagebrium reduced carotid AI by 37% ($P = 0.007$) and augmented pressure (16.4 ± 10 to 9.6 ± 9 mmHg; $P < 0.001$). Heart rate, arterial pressures, and ArtD, were unchanged. FMD increased from 4.6 ± 1.1 to 7.1 ± 1.1% with alagebrium ($P < 0.05$), and was unrelated to altered shear stress or regional arterial distensibility. However, FMD change was inversely related to markers of collagen synthesis, p-selectin and intracellular cell adhesion molecule (all $P < 0.05$). Alagebrium-associated changes in plasma nitrite plus nitrate was inversely correlated with plasma matrix metalloproteinase 9 and type I collagen ($P = 0.007$).**

Conclusions **Alagebrium enhances peripheral artery endothelial function and improves overall impedance matching. Improved endothelial function correlates better with reduced vascular fibrosis and inflammation markers than with vessel distensibility. AGE-crosslink breakers may reduce cardiovascular risk in older adults by reduced central arterial stiffness and vascular remodeling.**
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Introduction

Decreased vascular distensibility and endothelial dysfunction are hallmarks of the aging process [1,2], and are associated with an increased risk of cardiovascular disease [3–6]. Both abnormalities often coexist and may be mechanistically inter-related [7]. Endothelial dysfunction reduces the synthesis of nitric oxide (NO) and other vasodilators, increasing smooth muscle tone and thus vessel stiffness. Alternatively, wall compliance may influence endothelial function, as revealed by in-vitro studies showing reduced nitric oxide synthase and Akt activation in endothelial cells exposed to pulse perfusion when cultured in less distensible conduits [8–10].

Another mechanism whereby vascular wall stiffness and endothelial dysfunction may be linked is by the formation of advanced glycation endproducts (AGE) [11]. These protein–glucose crosslinks accrue over time and are exacerbated by elevated glucose and oxidant stress. They alter vascular structure and function by three basic mechanisms [12–14]. First, the accumulation of AGE crosslinked collagen in the arterial wall can reduce compliance and impact NO signaling by limiting stretch.

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Second, AGE can quench NO [15] and can inhibit flow-mediated NO release *in vitro* [16], promoting adhesion and the uptake of oxidized low-density lipoproteins to stimulate inflammatory changes in the extracellular matrix [17]. Third, AGE interacts with receptors (RAGE) to stimulate oxidant stress and upregulate cell surface adhesion molecules and cytokines stimulating vascular inflammation, remodeling and atherogenesis [18,19].

Advanced glycation crosslink breakers, such as alagebrium chloride (ALT-711), have previously been shown to reduce vascular stiffening in aged and hypertensive experimental models [20], and in older adults with isolated systolic hypertension [21]. To date, there are no data testing whether such therapy enhances endothelial function in humans, or if any such effect relates to improved arterial distensibility or to other potential mechanisms.

To examine this, this study tested the influence of alagebrium on brachial flow-mediated dilation (FMD) in older adults with systolic hypertension, and investigated its relationship to serum markers of collagen turnover and vascular inflammation, and measures of regional distensibility. Alagebrium improved endothelial function independent of regional changes in vessel wall compliance, and correlated with a decline in blood markers of fibrosis, vascular remodeling and inflammation.

Methods

Study population

Thirteen subjects (nine men, four women) recruited from the Johns Hopkins Cardiology Outpatient Clinic were studied. All had isolated systolic hypertension (systolic blood pressure ≥ 140 and < 200 mmHg, diastolic blood pressure ≤ 90 mmHg, or pulse pressure > 60 mmHg) and had a mean age and body mass index of 65 ± 2.4 years and 29.5 ± 1.4 kg/m^2, respectively. Subjects could be taking antihypertensive medications, but a stable regimen was required for at least 1 month before enrollment and throughout the study. Only one subject had diabetes, which was diet controlled. Exclusion criteria were: aortic stenosis; atrial fibrillation; a history of coronary artery disease, stroke or peripheral vascular disease; left ventricular ejection fraction less than 55%; aspartate aminotransferase and alanine aminotransferase more than twice the normal limit; creatinine greater than 2.0 mg/dl; cigarette smoking; previous exposure to alagebrium or other investigational drugs within 1 month; inability to provide voluntary informed consent, and inability to abstain from caffeine, food, alcohol, anti-oxidants, or nicotine-containing products for at least 8 h before study visits.

Protocol

The study design was a single-blind, phase 2a placebo run-in single-arm study. Subjects were given color and sized-matched placebo tablets (three tablets taken twice a day) for 2 weeks followed by alagebrium chloride (210 mg twice a day taken as three 70 mg tablets twice a day) for 8 weeks, and were blinded as to placebo versus study drug tablets. The dose was chosen based on a previous clinical trial demonstrating an improvement in total arterial compliance [21]. Alagebrium (ALT-711; 4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride; IND# 59,807) and placebo were supplied by Alteon, Inc. (Parsippany, New Jersey, USA). Study medication compliance was assessed by pill counts at 2–4-week intervals.

Studies, including physical examination, electrocardiogram, blood laboratories, and symptom questionnaires were performed between 0800 and 1100 h on weeks 0, 2, 6, 10 and at follow-up (week 12). Blood pressure was assessed manually in the non-dominant arm with patients seated quietly for 10 min (mean of last three out of five readings, each separated by 1 min). On week 2 and 10 visits, subjects were further evaluated by applanation tonometry and ultrasound imaging to assess endothelial function (brachial artery FMD) and vascular distensibility.

Brachial flow-mediated dilation

Brachial artery FMD was performed as previously reported [22]. Subjects lay quietly supine for 20 min, and the non-dominant brachial artery was then imaged by a 15 mHz vascular probe (Agilent) and echocardiography (Hewlett Packard Sonos 5500). Distal artery occlusion was performed for 5 min by inflating a blood pressure cuff more than 20 mmHg above systolic blood pressure. Brachial artery gated images and Doppler flow were recorded for 3 min after distal cuff deflation. FMD was calculated as a percentage change in brachial arterial diameter from baseline to the maximal dilation within the 3 min post-deflation. Changes in arterial velocity and shear stress were also calculated as a percentage change from baseline to peak velocity after cuff deflation for brachial vasoreactivity studies before and after 8 weeks of alagebrium administration. Pulsatile shear stress was calculated using a modification of Womersely's formula previously reported by our group [23]. All images were stored on optical discs for assessment using Brachial Analyzer (Medical Imaging Application; Iowa City, Iowa, USA) by a reader blinded to treatment condition. Our laboratory and others have previously reported on the consistent reproducibility of the FMD technique employed in this study when performed on two subsequent days [22,24].

Measurement of vascular distensibility

Arterial stiffness was assessed using an automated device (Colin VP2000) that simultaneously acquired applanation tonometry of the carotid artery, phonocardiography, and oscillometric brachial pressure, allowing recreation of the mean carotid waveforms for an evaluation of pressure augmentation, a verified measure of arterial stiffness and impedance mismatch. The carotid augmentation index (AI), a marker of reflected wave magnitude and timing, was

calculated from digitized (1.2 kHz) tonometry waveforms and analysed by custom software using established algorithms [25]. Central blood pressure was estimated from carotid tonometry calibrated to mean and diastolic brachial blood pressure. Stroke volume was determined from the product of the aortic cross-sectional area and the velocity–time integral in the aortic root assessed by echocardiography with pulsed wave Doppler. Regional brachial artery distensibility was assessed from systolic and diastolic dimensions and pressures using formulae for elastic modulus and stiffness index as previously described [26,27].

Serum markers of collagen metabolism, inflammation and endothelial function

To determine the effect of alagebrium on collagen metabolism, serum was collected before and after alagebrium treatment and analysed for types I and III collagen synthesis markers [C-terminal procollagen type I propeptide (PICP), procollagen type I N-terminal propeptide (PINP), and N-terminal procollagen type III propeptide (PIIINP)]. These collagen markers were measured by radioimmunoassay (Quidel, Mountain View, California, USA, PICP; and DiaSorin, Stillwater, Minnesota, USA, all others). These markers have been found to correlate with arterial and ventricular fibrosis, as well as hypertension, and therapies such as angiotensin-converting enzyme inhibition that reduce vascular fibrosis, remodeling, and stiffness, and decrease levels of collagen synthesis markers [16,28–31]. To elucidate further the potential mechanism of action of alagebrium, serum levels of the matrix metalloproteinase (MMP type 9; gelatinase B), were measured before and after alagebrium therapy by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota, USA). AGE can also impact on inflammatory markers such as transforming growth factor beta 1 (TGF-β_1), p-selectin and intracellular cell adhesion molecule (ICAM) type 1 and vascular cell adhesion molecule (VCAM) type 1 [18,19,32]. Therefore, we also assessed serum levels of these proteins before and after alagebrium treatment by enzyme-linked immunosorbent assay (R&D Systems). Finally, serum nitrite (NO^{2-}) plus nitrate (NO^{3-}), a measure of nitric oxide production, were measured before and after alagebrium therapy by the Griess reaction.

Ethical and statistical information

The protocol was approved by the Johns Hopkins Institutional Review Board, and all subjects gave voluntary informed consent to participate. The study is registered on the Clinical Trials Registry (www.clinicaltrials.gov) as NCT00277875. The authors had full access to the data and take full responsibility for its integrity, and all authors have read and agree to the manuscript as written.

This study was powered to detect a 2% increase in FMD, the primary outcome, using a two-tailed alpha level of 0.05 and an 80% beta error rate. Comparison of nominal variables was performed using a paired Student's *t*-test before and after treatment. Univariate linear regression was used to assess correlations between variables.

Table 1 Change in measures of vital signs, endothelial function and indices of arterial stiffness before and after treatment with alagebrium chloride (mean ± SEM)

Variable	Baseline	Post-treatment	*P* value
Systolic blood pressure (mmHg)	146 ± 3.9	142 ± 3.5	0.29
Diastolic blood pressure (mmHg)	82 ± 2.8	82 ± 3.6	0.91
Pulse pressure (mmHg)	64 ± 2.4	60 ± 2.4	0.12
Mean arterial pressure (mmHg)	103 ± 3.0	102 ± 3.3	0.68
Heart rate (bpm)	63 ± 2.5	63 ± 2.4	0.92
Augmentation index (%)	0.31 ± 0.04	0.20 ± 0.05	0.007
Carotid augmented pressure (mmHg)	16.5 ± 2.8	9.6 ± 2.6	0.0008
Brachial distensibility			
Elastic modulus (mmHg)	2057 ± 257	2035 ± 232	0.93
Stiffness index	18.3 ± 2.4	18.7 ± 2.2	0.87

$N = 10$.

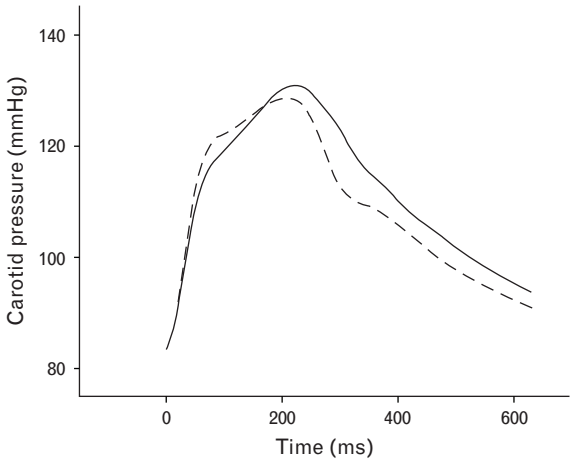
Results

Baseline and post-alagebrium treatment values of arterial properties and hemodynamics are provided in Table 1. Alagebrium treatment did not significantly alter left ventricular wall thickness, cardiac output, stroke volume, or systolic and diastolic function (data not shown). There were no changes in electrocardiogram rhythm, PR, QRS and QTc intervals or ST-T waves, and no significant adverse events were associated with alagebrium treatment, with the exception of one subject who died of 'natural causes – atherosclerotic heart disease' 63 days after the last dose of study medication, which was adjudicated as 'possibly related' to the study medication. All subjects were taking consistent doses of at least one antihypertensive medication at the time of enrollment, with the exception of one subject who had not been treated for hypertension. Seven subjects were taking beta-blockers, five were taking diuretics and two subjects each were taking an angiotensin receptor blocker, a calcium antagonist or an alpha antagonist. No interaction was seen between the type of antihypertension medication and the effects of alagebrium on the vasculature.

Vascular distensibility

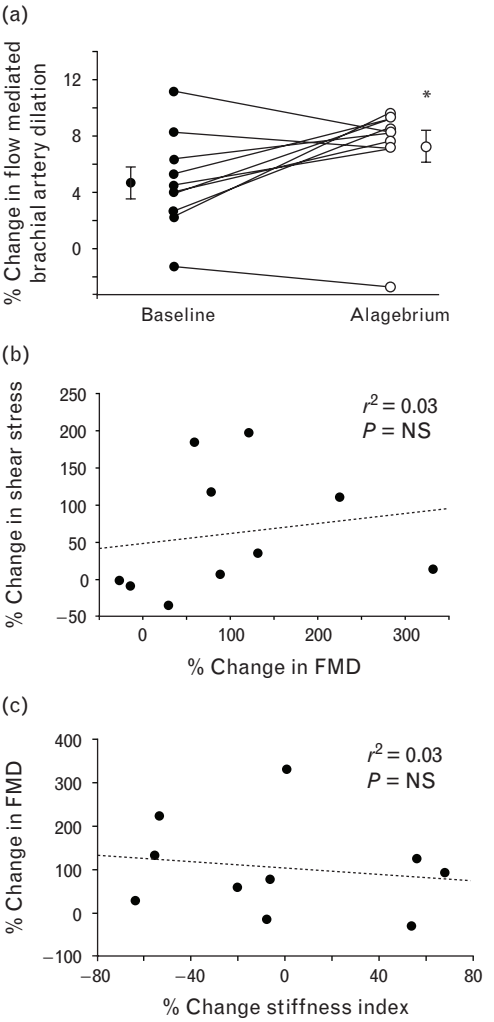
Central arterial impedance mismatch, reflected by a 37% fall in the carotid AI and a 42% decline in pressure augmentation, was significantly improved with alagebrium therapy ($P < 0.01$; Table 1). Figure 1 shows the effect of chronic alagebrium treatment on the average carotid pressure waveform. Measures of brachial artery vascular stiffness, for example, brachial artery distensibility (elastic modulus and stiffness index), were unchanged by alagebrium therapy. In addition, treatment with alagebrium did not alter resting brachial systolic, diastolic, mean or pulse pressure or systemic vascular resistance.

Fig. 1



Carotid artery pressure waveforms measured by applanation tonometry both before and after 8 weeks of treatment with alagebrium chloride. Data curves reflect an average result from all patients entered into the study. Alagebrium lowered the late systolic pressure augmentation and thus the augmentation index. —— Baseline; – – – post-alagebrium.

Fig. 2



(a) Flow-mediated dilation expressed as a percentage change in brachial artery diameter measured at baseline and after 8 weeks of alagebrium treatment. Mean percentages pre and post-therapy ± SEM are shown. $^*P < 0.05$ versus baseline. (b) Lack of correlation between percentage change in flow-mediated dilation (FMD) versus percentage change in shear stress before and after alagebrium treatment. (c) Lack of correlation between percentage change in FMD and percentage change in stiffness index (a measure of vascular distensibility) of the brachial artery before and after alagebrium therapy.

Flow-mediated dilation

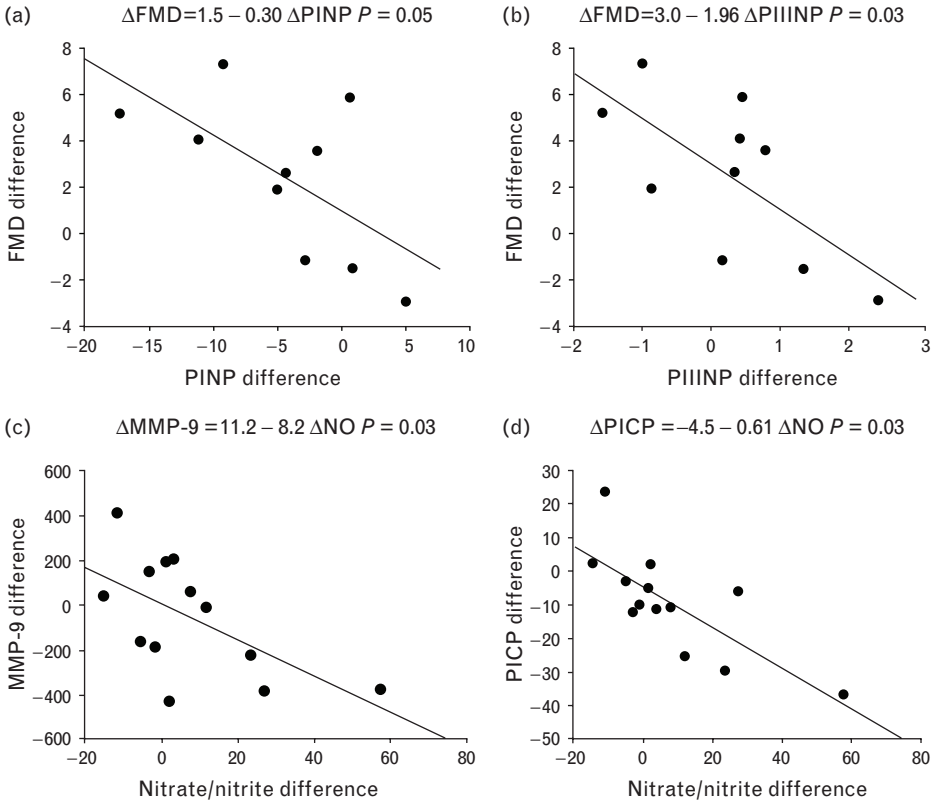
FMD could not be assessed in three subjects because of motion artifact or a lack of imaging clarity of the endothelial wall, as determined by a blinded independent reader. Results for the remaining 10 subjects are presented in Fig. 2a. Alagebrium improved FMD from 4.64 ± 1.07 to $7.10 \pm 1.13\%$ ($P = 0.047$), a mean increase of $102 \pm 34\%$ ($P = 0.02$). Baseline arterial diameters were not significantly different before (3.96 ± 0.23 mm) and after (3.97 ± 0.22 mm) alagebrium therapy ($P = 0.90$). The flow-mediated change in absolute arterial diameter increase from 0.18 ± 0.05 mm at baseline to 0.27 ± 0.05 mm after treatment ($P = 0.068$). The mean flow velocity rose by 58.4 ± 9.7 at baseline, compared with $77.3 \pm 9.5\%$ after alagebrium ($P = 0.03$). This change was consistent with the augmentation of pulsatile shear stress after cuff deflation of 61.9% with alagebrium ($P = 0.04$). As shear stress is a major stimulant for vessel dilation, we compared individual changes in FMD. and estimated pulsatile shear stress before and after alagebrium therapy (Fig. 2b). However, these parameters were not correlated supporting enhanced endothelial response rather than altered mechanical input as the underlying mechanism. To test whether FMD was related to changes in regional brachial distensibility, we also compared individual changes in these two variables (Fig. 2c). Once again, no correlation was observed.

In contrast to mechanical properties (i.e. vessel compliance and shear stress), FMD significantly correlated with decreases in the serum levels of collagen synthesis markers PINP ($r = 0.63$, $P = 0.05$) and PIIINP ($r = 0.68$, $P = 0.03$; Fig. 3a and b) as well as inflammatory markers p-selectin ($r = 0.67$, $P = 0.03$) and ICAM ($r = 0.62$, $P = 0.05$).

Effects of alagebrium treatment on matrix/inflammatory markers

C-terminal procollagen type I propeptide (PICP), a marker of fibrosis, declined from 48.2 ± 4.6 to 38.9 ± 4.1 ng/ml ($P = 0.05$) after alagebrium treatment. In contrast, alagebrium therapy was associated with a borderline significant rise in total serum nitrite plus nitrate ($P = 0.11$). Examined on an individual basis, however, changes in nitrite plus nitrate strongly correlated with a decline in serum PICP ($r = 0.76$, $P = 0.003$) and serum MMP-9 ($r = 0.60$, $P = 0.03$; Fig. 3c and d). Changes

Fig. 3



Relationship between the change in flow-mediated dilation (FMD) and the change in serum markers of fibrosis (a) and inflammation (b) comparing baseline to 8 weeks after alagebrium treatment shown as simple linear regressions. Lower panels demonstrate the change in total plasma nitrite plus nitrate and metalloproteinase 9 (MMP-9) (c), or C-terminal procollagen type I propeptide (PICP) (d) between baseline and post-alagebrium treatment. These simple linear regressions show increases in nitrite plus nitrate inversely correlated with these matrix/fibrosis markers.

in serum TGF-β_1 (26.5 ± 0.7 to 20.6 ± 2.8 ng/ml) and VCAM-1 (821 ± 33 to 786 ± 28 ng/ml) achieved borderline significance (both $P = 0.08$) with alagebrium treatment.

Discussion

This study provides the first evidence that an AGE crosslink breaker enhances endothelial function as reflected by FMD in humans, and helps identify mechanisms likely to be related to this effect. The rise in FMD was uncorrelated with regional changes in artery distensibility or shear stress, but rather with a reduction in blood markers of vascular fibrosis and remodeling. Despite the lack of an alagebrium-related change in brachial artery distensibility, alagebrium treatment improved overall impedance matching, as evidenced by a reduction in carotid wave reflection. These data indicate that AGE crosslink breakers can improve vascular endothelial function in humans by a mechanism independent of altered structural stiffness.

AGE forms in long-lived proteins such as collagen in the vascular wall [33], and the resulting crosslinked collagen is less amenable to degradation by metalloproteinases, thereby contributing to arterial wall thickening and reduced distensibility [34]. Alagebrium has previously been shown to lower central arterial stiffness in non-human primates [20] and aged humans with isolated systolic hypertension [21]. Consistent with these results, our study demonstrated a reduction in carotid AI with alagebrium therapy. This decline in central pressure augmentation can indirectly reflect arterial stiffening, but is also quite sensitive to peripheral vascular properties, which in turn can depend on endothelial function. AI is a risk factor for cardiovascular disease [35], so the change observed with alagebrium treatment may be beneficial.

The effects of alagebrium treatment on markers of collagen synthesis, fibrosis, and inflammation have not previously been reported in humans. We observed declines in several markers of collagen synthesis, such as PINP and PIIINP, and inflammatory markers, such as p-selectin, which correlated with improved FMD. These data support a molecular/signaling rather than a mechanical mechanism underlying improved endothelial

function by alagebrium. The strongest correlations were observed between declines in PICP and MMP-9 levels and increases in plasma nitrate plus nitrite as a result of alagebrium treatment. Both PICP and MMP-9 correlate with arterial stiffness, endothelial dysfunction, atherogenesis and plaque vulnerability [36–38], and can serve as markers of tissue fibrosis and matrix turnover. AGE formation can reduce endothelial NO synthesis, which in turn leads to enhanced TGF-β_1 and MMP-9 activity followed by increased fibrosis. Alternatively, increasing NO inhibits TGF-β_1 activation and vascular remodeling [39,40]. AGE can upregulate TGF-β_1 [19], and although we found only a borderline decline in this enzyme with alagebrium treatment, the data overall seem most consistent with a mechanism linking endothelial function to this type of signaling.

AGE also influences vascular function by binding to a group of immunoglobulin superfamily receptors, RAGE [41]. AGE–RAGE interaction stimulates inflammatory responses by the production of oxidant radicals, nuclear factor kappa B, proinflammatory cytokines, growth factors, and vascular adhesion molecules (IL-6, tumor necrosis factor α, TGF-β_1 and VCAM) [32,42,43]. This can enhance vascular permeability and also result in endothelial dysfunction [42,44]. Animals treated with a soluble RAGE or RAGE IgG to block this ligand-receptor signaling show reduced VCAM, MMP, tissue factor, and macrophage chemotactic factor [32,45,46]. Our data, which revealed an inverse correlation between FMD improvement and serum ICAM and p-selectin levels, and a trend towards reduced VCAM levels, support a potential role of this mechanism in humans.

AGE–RAGE interactions also depress endothelial NO responsiveness to mechanical and chemical stimuli, as revealed in experimental studies *in vivo* [15,47,48] and *in vitro* [16]. For example, endothelial cells cultured on an AGE collagen matrix have reduced shear-stress-mediated NO release [16]. Such vascular effects can be blunted by treatment with the AGE inhibitor aminoguanidine. Brachial artery endothelial FMD is thought to be largely caused by shear stress-induced NO release, so the current findings are consistent with attenuated AGE–RAGE interaction rather than a compliance-dependent improvement in NO release [10].

Our study is limited in that it utilized a non-placebo-controlled single-blind design, and it is not possible to rule out a placebo effect on FMD. Importantly, all analyses were performed blinded as to temporal sequence, and patients were all treated with a blinded placebo run-in period to acclimate them to the clinical setting, investigators, and procedures. Moreover, the consistency of baseline arterial measures and a 102% increase in FMD, which is more than two standard deviations beyond that expected from a placebo effect based on previous studies of FMD, suggest a true therapeutic effect [22,24].

Improved endothelial FMD by an AGE crosslink breaker provides further support for the potential benefits of drugs such as alagebrium for the treatment of human vascular pathobiology. These results further support an underlying mechanism whereby alagebrium treatment can enhance NO generation correlated with markers of matrix formation/turnover. Whereas recent clinical trials using alagebrium to target systolic hypertension have not supported benefits [49], a lack of systolic pressure change may not imply a lack of biological effects. Accordingly, an improvement in measures of central vascular compliance was seen after alagebrium therapy in this study and in an earlier study [21] in the absence of any significant alteration in brachial blood pressures. As endothelial dysfunction, itself, is associated with increased cardiovascular risks, the reversal of AGE and AGE–RAGE interactions may still prove an important therapeutic target.

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