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ARTICLE

Delayed intervention with AGE inhibitors attenuates the progression of diabetes-accelerated atherosclerosis in diabetic apolipoprotein E knockout mice

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Abstract
Aims/hypothesis Formation of AGEs is increased in the diabetic milieu, which contributes to accelerated atherogenesis. We studied whether delayed treatment with AGE-inhibiting compounds, alagebrium chloride and pyridoxamine dihydrochloride, affected established atherosclerosis in experimental diabetes in comparison with the angiotensin-converting enzyme inhibitor, quinapril.
Methods Streptozotocin-induced diabetic male *Apoe*$^{-/-}$ mice (n=24 per group) received, by oral gavage, from week 10 to 20 of diabetes: no treatment; alagebrium (1 mg kg^{-1} day^{-1}); pyridoxamine (1 g/l in drinking water); or quinapril (30 mg kg^{-1} day^{-1}). Atherosclerotic lesion area (en face analysis) was evaluated for all groups.
Results Delayed intervention with alagebrium decreased plaque area in the diabetic *Apoe*$^{-/-}$ mice compared with untreated mice (total plaque area: alagebrium 10.6±1.6%, untreated, 15.1±1.5%, p<0.05). This anti-atherosclerotic effect was comparable with that achieved with quinapril (quinapril 8.4±1.4%, vs untreated, p<0.05). Pyridoxamine also attenuated plaque development in diabetic mice (5.7±1.2% vs untreated 11.9±1.1%, p<0.05). The anti-atherosclerotic effect conferred by alagebrium and quinapril was associated with a significant reduction in vascular oxidative stress and circulating AGEs and methylglyoxal, although preformed AGEs were not removed from the vascular wall with either delayed intervention.
Conclusions/interpretation Inhibition of AGE accumulation, using a delayed intervention with alagebrium or pyridoxamine, significantly attenuated the progression of established diabetes-associated atherosclerosis, similar to results obtained with quinapril. These findings provide further evidence that blockade of AGE-mediated pathways may present a novel therapy for the prevention of atherosclerosis in diabetes.

Keywords ACE inhibition · Alagebrium · Atherosclerosis · Diabetes mellitus · Mouse model · Pyridoxamine

Abbreviations

ACE	Angiotensin-converting enzyme
AT1a	Angiotensin receptor type 1a
AT2	Angiotensin receptor type 2
GHb	Glycated haemoglobin
MCP-1	Macrophage chemoattractant protein 1
NHMRC	National Health and Medical Research Council

A. M. D. Watson and A. Soro-Paavonen contributed equally to this study.

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RAGE	Receptor for AGEs
RAS	Renin–angiotensin system
sRAGE	Soluble receptor for AGEs
VCAM1	Vascular cell adhesion molecule 1

Introduction

Diabetes is associated with accelerated development and progression of atherosclerosis, which contributes to the increased burden of cardiovascular disease in patients with diabetes. Although a number of different factors contribute to accelerated atherosclerosis in diabetes, recent data show that the accumulation of AGEs may have a significant role [1]. AGEs are post-translational modifications of proteins, DNA or lipids formed in vivo under the influence of carbonyl stress. AGE modifications have the potential to alter the structure and/or function of proteins, as well as to activate pro-atherogenic pathways including oxidative stress, inflammation [2] and the renin–angiotensin system (RAS) [3, 4].

In this study we explore the anti-atherosclerotic effect of two chemically different inhibitors of AGE accumulation, alagebrium chloride (ALT or ALT-711) and pyridoxamine in the diabetic apolipoprotein E knockout mouse (*Apoe*$^{-/-}$), a model of accelerated atherosclerosis [5–7]. Previous studies have shown that when given directly after the induction of diabetes, treatment with AGE inhibitors was able to attenuate atherosclerosis development [8]. It has been shown that diabetic *Apoe*$^{-/-}$ mice with established atherosclerosis had decreased plaque area when treated with the decoy AGE receptor, soluble receptor for AGE (sRAGE) [5]. However, this is the only study to examine the effects of anti-atherosclerotic treatments for the secondary prevention of lesion development in the setting of established atherosclerosis.

Thus, in this study we aimed to examine whether alagebrium and pyridoxamine are able to attenuate the progression of atherosclerosis in animals with established diabetes-associated atherosclerosis and to compare these actions with that of the angiotensin-converting enzyme (ACE) inhibitor quinapril.

Methods

Experimental model

Male *Apoe*$^{-/-}$ mice (backcrossed 20 times to a C57BL/6 background; Animal Resource Centre, Canning Vale, WA, Australia) were rendered diabetic at the age of 6 weeks via five daily intraperitoneal injections of streptozotocin (Boehringer Ingelheim, Mannheim, Germany) 55 mg kg^{-1} day^{-1}, resulting in insulin deficiency [7]. After exposure to untreated diabetes for 10 weeks, mice (n=24 per group) were randomised to receive, from 10 to 20 weeks after the induction of diabetes (weeks 16–26 of age), via daily oral gavage: (1) no treatment; (2) the anti-AGE agent alagebrium chloride (4,5-dimethyl-3-[2-oxo-2-phenylethyl]-thiazolium chloride; Synvista Therapeutics, Montvale, NJ, USA), 1 mg kg^{-1} day^{-1}; or (3) quinapril (Accupril; Pfizer Australia, West Ryde, NSW, Australia), 30 mg kg^{-1} day^{-1}. In addition, untreated control and diabetic animals were killed after 10 weeks diabetes in order to determine the plaque area at this stage. In a separate study this same protocol was used to treat animals with pyridoxamine (pyridoxamine dihydrochloride; BioStratum, Durham, North Carolina, USA), 1 g/l in drinking water.

The mice were housed at the Precinct Animal Centre, Baker Heart Research Institute, and studied according to National Health and Medical Research Council (NHMRC) guidelines after ethics approval from the Alfred Medical Research Precinct Animal Ethics Committee in line with international standards.

Mice were allowed access to standard mouse chow (Specialty Feeds, Glen Forrest, WA, Australia) and water ad libitum. Mice were killed using an intraperitoneal injection of Euthatal (100 mg/kg) (Delvet Limited, Seven Hills, NSW, Australia), followed by exsanguination by cardiac puncture. Excised aortas were placed in 10% neutral buffered formalin and the lesion areas were quantified before the aortas were embedded in paraffin for immunohistochemical analysis. In a subset of animals, aortas were snap frozen in liquid nitrogen and stored at −70°C for subsequent RNA extraction. At the conclusion of the study, glycated haemoglobin (GHb) was measured by high-performance liquid chromatography [9]. Total plasma cholesterol, HDL-cholesterol and triacylglycerol concentrations were measured in nine to ten mice per group using a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser (Beckman Coulter Diagnostics, Australia). LDL-cholesterol was calculated using the Friedewald formula.

Plaque area

The plaque area was quantified as described previously by Calkin et al. and Candido et al. [6, 7] In brief, aortas were cleaned of excess fat under a dissecting microscope and subsequently stained with Sudan IV-Herxheimer's solution (0.5% wt/vol.) (Gurr; BDH, Poole, UK). Aortas were dissected longitudinally, divided into arch, thoracic, and abdominal segments, and pinned flat onto wax. Images were acquired with a dissecting microscope equipped with an Axiocam camera (Zeiss, Heidelberg, Germany). Total and segmental plaque areas were quantitated as a percentage area of aorta stained (Adobe Photoshop, version 7.0).

Tissue was subsequently embedded in paraffin and sections cut for immunohistochemical analysis.

Real-time RT-PCR

Total RNA was extracted from whole aorta by homogenising (Polytron PT-MR2100; Kinematica, Littau-Lucerne, Switzerland) in Trizol (Life Technologies, Rockville, MD, USA). Total RNA was then DNAse-treated (DNA removal kit; Ambion, Austin, TX, USA) and cDNA was synthesised by reverse transcription (Pierce Biotechnology, Rockford, IL, USA). Quantitative real-time RT-PCR was performed using the Taqman System on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and analysed using a software detection system (SDS version 1.9) software. Gene expression was normalised to 18S rRNA (Applied Biosystems). Detailed information on probes is provided in Electronic supplementary material (ESM) Table 1. For each analysis, nine to 13 animals per group were used.

Immunohistochemistry

Formalin-fixed paraffin sections of aortas prepared previously were used for trichrome staining (as per standard protocol), or were stained using antibodies: rabbit anti-nitrotyrosine (1:50; Chemicon, Temecula, CA, USA), goat anti-receptor for AGE (RAGE, 1:400; Biologo, Kiel, Germany); antibody against the macrophage marker F4/80 (rat anti-F4/80, 1:50; Abcam, Cambridge, UK); glycated serum albumin antibody (1:250; kindly provided by M. Coughlan, Diabetes Complications Division, Baker IDI Heart and Diabetes Institute, Melbourne, VIC, Australia); or rabbit anti-carboxymethyllysine (1:1,000 Abcam). AGE-serum albumin antibody was pre-absorbed against BSA (Sigma-Aldrich, St Louis, MO, USA) for 30 min before use. Paraffin sections were dewaxed in xylene and hydrated. Slides were washed with distilled water and Tris-buffered NaCl (TBS; pH 7.6). They were then incubated with 0.3% hydrogen peroxide in TBS for 20 min. Subsequently, the slides were washed with distilled water and incubated with protein-blocking agent (Lipshaw-Immunon, Pittsburg, PA, USA) or 0.5% skimmed milk powder in TBS for 30 min. Additional blocking of endogenous avidin/biotin was used for nitrotyrosine staining (Avidin-Biotin Blocking Kit; Vector Laboratories, Burlingame, CA, USA). Sections were incubated with primary antibody prepared in TBS overnight at 4°C. After washing (TBS and TBS+Tween 20), slides were incubated with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 10 min at room temperature. Additionally, sections incubated with anti-F4/80 antibody were incubated in amplification reagent and strep-HRP (Dako catalysed signal amplification (CSA) kit; Dako North America, Carpinteria, CA, USA). All sections were then washed and stained with avidin–biotin horseradish peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories). Positive staining was visualised with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and counterstained in Mayer's haematoxylin for 1 min, followed by Scott's tap water for 15 s. All experiments included negative control slides, in which the primary antibody had been omitted. Digital analysis of percentage of area stained (excluding adventitia) was performed as described previously by Soro-Paavonen et al. [10].

Assessment of plasma AGE levels

Plasma albumin fluorescence measurements were performed as described previously [11].

Plasma methylglyoxal measurements were performed using 20 μl plasma samples which were diluted 1:5 with ammonium sulphate solution before adding chloroform (1:2 dilution). Samples were mixed, spun down and the resulting organic/aqueous interface precipitate removed by decanting the aqueous supernatant fraction. 5,6-Diamino-

Table 1 Metabolic variables of the groups at week 20

Variable	Group			
	Control	Diabetic	Diabetic/ALT	Diabetic/quinapril
n	10	10	10	10
GHb (%)[a]	4.1±0.3	15.3±0.8***	16.5±0.6***	17.7±0.7***
Plasma glucose (mmol/l)	11.0±0.7	29.0±1.4***	33.0±1.3***	31.4±2.6***
Body weight (g)[a]	31.0±0.4	25.1±0.5***	23.0±0.4***	23.0±0.5***
Systolic blood pressure (mmHg)	124.0±1.8	127.0±2.6	120.9±1.5‡	113.7±3.4†
Total cholesterol (mmol/l)	13.7±0.9	23.0±2.0***	24.0±2.6***	28.1±3.5***
HDL-cholesterol (mmol/l)	2.7±0.2	3.0±0.3	3.4±0.4	4.6±0.7
LDL-cholesterol (mmol/l)	10.3±0.8	19.0±1.9***	20.0±2.5***	23.1±3.0***
Triacylglycerol (mmol/l)	1.7±0.2	2.2±0.2	1.6±0.2	1.7±0.3

Data shown as means±SEM

[a] Performed in 24 animals per group

***$p<0.001$ vs control *Apoe*$^{-/-}$ group; †$p<0.05$ vs diabetic *Apoe*$^{-/-}$ group; ‡$p<0.005$ vs diabetic *Apoe*$^{-/-}$ plus quinapril group

ALT, alagebrium

2,4-dihydroxypyrimidine sulphate (Sigma-Aldrich), 7 mmol/l, was then added to the supernatant fraction before heating to 60°C for 60 min, cooling in ice, then spinning again. The resulting reacted plasma solution was run through HPLC with a 10 nmol/l citrate buffer/methanol (pH 7.0) mobile phase, C18 stationary phase (Waters 'Atlantis' T3 4.6×150 mm 3 μm column, Dublin, Ireland) with 330/500 nm excitation/emission at 25°C and methylglyoxal derivative eluting at 28 min.

Statistical analysis

Data were analysed by ANOVA using SPSS 15.0 software. Post hoc comparisons were made among the various groups using Fisher's least significant difference method. Data are expressed as mean±SEM unless otherwise specified. $p<0.05$ was considered to be statistically significant.

Results

Metabolic variables

The induction of experimental diabetes was associated with significant increases in GHb and plasma glucose levels (Table 1). Untreated diabetic animals in the second study (delayed intervention treatment with pyridoxamine) also had significantly elevated levels of GHb compared with non-diabetic controls (16.2±1.0 [$n=8$] compared with 3.4± 0.3% [$n=8$] respectively; $p<0.05$). Treatment with alagebrium, quinapril (Table 1) or pyridoxamine (GHb 17.1± 0.7%, $n=7$) did not significantly affect glycaemic control in diabetic mice. Plasma concentrations of total cholesterol and LDL-cholesterol levels were also significantly higher in untreated diabetic animals (Table 1). These and other variables, such as body weight, were not significantly changed by alagebrium or quinapril treatment; however, delayed treatment with quinapril significantly decreased systolic blood pressure (Table 1).

Plaque area

Effect of delayed alagebrium or quinapril treatment Already after 10 weeks of diabetes, there was a significantly increased plaque area in diabetic compared with control *Apoe*$^{-/-}$ mice (total plaque area 3.1±0.3 vs 0.7±0.2%, compared with 0.7 ±0.2% respectively; $p<0.05$) (Fig. 1a–d). After 20 weeks of diabetes, the plaque area had increased fivefold in diabetic animals (total plaque area 15.1±1.5% compared with 4.3± 0.4% in non-diabetic controls) (Fig. 1). The diabetes-associated increase in plaque area was significantly attenuated in diabetic *Apoe*$^{-/-}$ mice treated with alagebrium (10.7± 1.6% total aortic plaque area) to a similar degree as that seen with quinapril treatment (8.4±1.4% total plaque area)

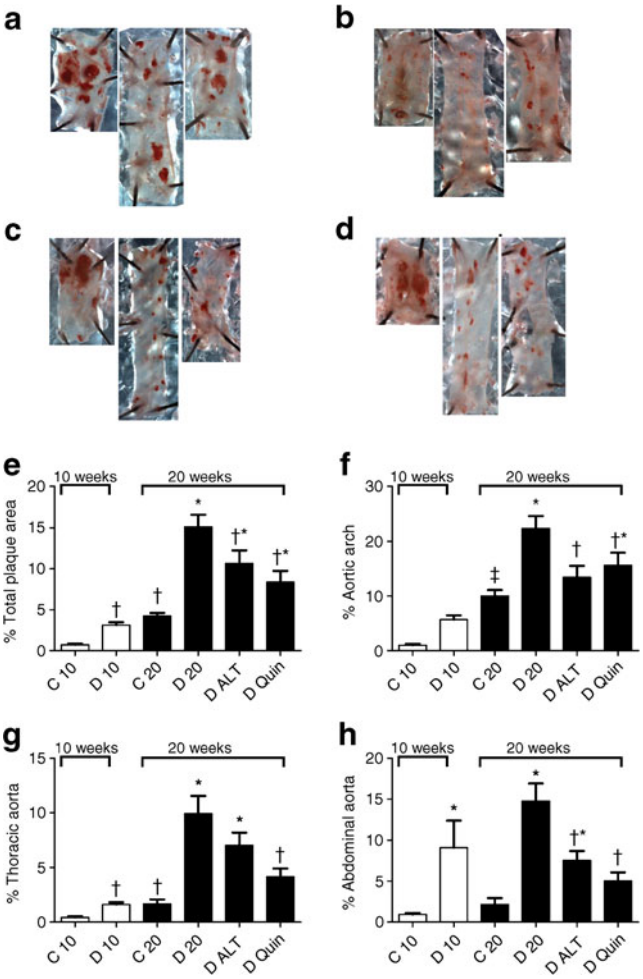

Fig. 1 **a**–**d** Representative photomicrographs of aortas at 20 weeks from: untreated diabetic mice (**a**); untreated non-diabetic control mice (**b**); diabetic mice+alagebrium 1 mg/kg for weeks 10–20 (**c**); diabetic mice+quinapril 30 mg/kg for weeks 10–20 (**d**). Lesions are shown in red after Sudan IV staining. **e**–**h** Quantification of atherosclerotic plaque area (%) of aortic segments by the en face method in total aorta after 10 and 20 weeks of diabetes: (**e**) total aortic area; (**f**) aortic arch; (**g**) thoracic aorta; and (**h**) abdominal aorta. At 10 weeks $n=7$–8 per group, at 20 weeks $n=12$–15 per group. *$p<0.05$ vs duration-matched control mice at 10 and 20 weeks, $^{\dagger}p<0.01$ vs diabetic untreated mice at 20 weeks, $^{\ddagger}p<0.05$ vs control mice at 10 weeks. In **g**, $p=0.055$ for D 20 vs D alagebrium. C 10, control mice at 10 weeks; D 10, diabetic mice at 10 weeks; C 20, control mice at 20 weeks; D 20, diabetic mice at 20 weeks; D ALT, diabetic mice with alagebrium 1 mg/kg at 20 weeks; D Quinapril, diabetic mice+quinapril 30 mg/kg at 20 weeks

(Fig. 1). However, neither treatment fully arrested the progression of atherosclerosis when given as a delayed treatment from 10 to 20 weeks.

Effect of delayed pyridoxamine treatment In the second study, untreated diabetic *Apoe*$^{-/-}$ animals also had a significant increase in aortic plaque area after 20 weeks of diabetes (total plaque area 11.9±1.1% compared with 3.7± 0.9% in non-diabetic controls; Fig. 2). Delayed intervention

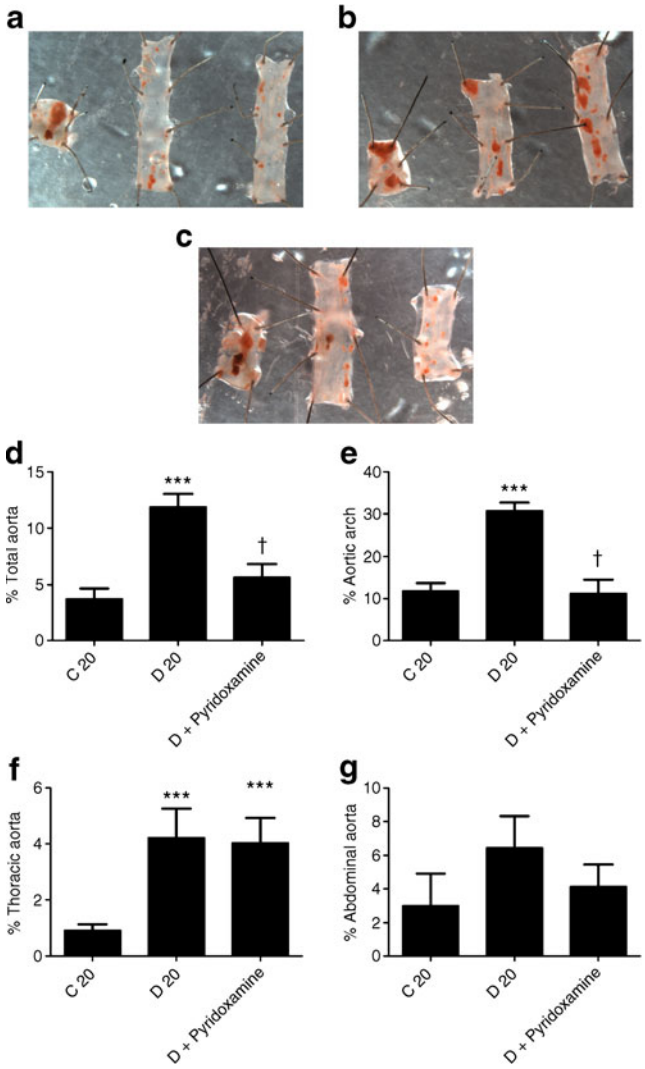

Fig. 2 **a** En face assessment of aortic plaque area in *Apoe*$^{-/-}$ mice which were untreated non-diabetic control (**a**), untreated diabetic (**b**), or diabetic animals treated with pyridoxamine from week 10 to 20 of diabetes (**c**). n=7–8 per group. **d–g** Percentage plaque area in: (**d**) total aortic area; (**e**) aortic arch; (**f**) thoracic aorta; and (**g**) abdominal aorta. Data shown as means±SEM. ***p<0.001 vs control *Apoe*$^{-/-}$ group; $^{\dagger}p$<0.05 vs diabetic *Apoe*$^{-/-}$ group. C 20, control mice at 20 weeks; D 20, diabetic mice at 20 weeks; D + pyridoxamine, diabetic animals treated with pyridoxamine from week 10–20 of diabetes

with pyridoxamine significantly attenuated plaque progression in diabetic animals (5.7±1.2% total plaque area; p< 0.05). Similar to delayed treatment with alagebrium and quinapril, pyridoxamine did not arrest plaque development.

AGE and RAGE

Diabetes was associated with a significant increase in the fluorescent modification of circulating albumin (Fig. 3a), a widely used marker of AGE modification. When used as delayed interventions, both alagebrium and quinapril were associated with a reduction in circulating fluorescent albumin, being significant in the quinapril-treated group (Fig. 3a). Diabetes was also associated with a significant increase the AGE precursor methylglyoxal (Fig. 3b). The diabetes-associated increase in plasma concentrations of methylglyoxal was also reduced by treatment with alagebrium and quinapril, with alagebrium being significantly more effective in reducing methylglyoxal than quinapril treatment (Fig. 3b). The induction of diabetes was also associated with increased accumulation of AGEs in the vascular wall of *Apoe*$^{-/-}$ animals, as detected by a polyclonal anti-AGE antibody (ESM Fig. 1). However, when used as delayed interventions, alagebrium and quinapril failed to significantly reduce the tissue levels of pre-formed AGEs, including carboxymethyllysine (ESM Figs 1, 2).

Diabetic *Apoe*$^{-/-}$ mice also showed significantly greater aortic expression of *Ager*, which encodes RAGE, and Cd36, which encodes the type B macrophage scavenger receptor CD36 antigen (Table 2). Aortic RAGE protein level, as assessed by immunostaining, was also significantly increased in diabetic *Apoe*$^{-/-}$ mice compared with non-

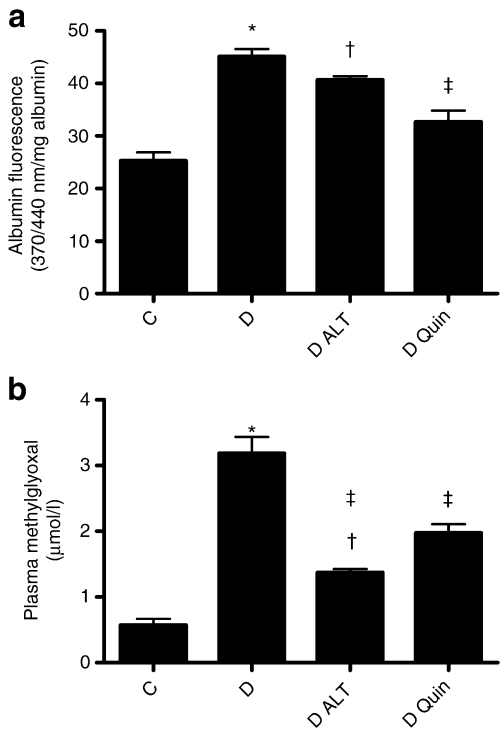

Fig. 3 (**a**) After 20 weeks significant increases in plasma albumin fluorescence (370/440 nm/mg albumin after 20 weeks, n=6 per group) and also (**b**) plasma methylglyoxal (n=4–7 per group) were attenuated by quinapril and alagebrium treatment. Thus both treatments significantly decrease circulating levels of AGEs. *p<0.05 vs control mice; $^{\dagger}p$<0.05 vs diabetic mice with delayed intervention with quinapril; $^{\ddagger}p$ <0.05 vs diabetic mice. C, non-diabetic control mice; D, diabetic mice; D ALT, diabetic mice with delayed intervention with alagebrium, 1 mg/kg; D Quin, diabetic mice with delayed intervention with quinapril, 30 mg/kg

Table 2 Gene expression of proatherogenic mediators in the aorta (current gene symbols in parentheses)

Variable	Group			
	Control	Diabetic	Diabetic/ALT	Diabetic/quinapril
n	11	11	11	7
Markers of oxidative stress and RAS				
p47phox (*Ncf1*)	1.0±0.2	5.9±2.3*	3.4±0.3$^{\ddagger a}$	9.7±2.1*
gp91phox (*Cybb*)	1.0±0.3	3.5±0.5*	3.6±0.6*	5.3±0.9*†
AT1A (*Agtr1a*)	1.0±0.2	5.1±1.3*	1.2±0.3†	1.2±0.4†
AT2 (*Agtr2*)	1.0±0.2	5.6±0.9*	3.0±0.4*†	3.0±0.4*†
ACE (*Ace*)	1.0±0.1	2.9±0.6*	2.3±0.4*	2.4±0.7
Mediators of inflammation and fibrosis				
VCAM1 (*Vcam1*)	1.0±0.2	4.3±0.7*	3.3±0.5*	2.9±0.4*†
MCP-1 (*Ccl2*)	1.0±0.3	6.4±1.3*	6.3±1.4*	6.8±1.3*
IL-1β (*Il1b*)	1.0±0.3	11.2±2.0*	6.7±1.0*†	5.4±1.2*†
AGE receptors				
RAGE (*Ager*)	1.0±0.1	4.3±1.1*	2.4±0.4^{b}	2.5±0.7
CD36 (*Cd36*)	1.0±0.2	2.1±0.4*	1.4±0.3	1.2±0.3

Data shown as means±SEM

*$p<0.05$ vs control *Apoe*$^{-/-}$ group; $^{\dagger}p<0.05$ vs diabetic *Apoe*$^{-/-}$ group; $^{\ddagger}p<0.05$ vs diabetic *Apoe*$^{-/-}$ plus quinapril group

$^{a}p=0.060$ for diabetic vs diabetic plus alagebrium; $^{b}p=0.057$ for diabetic vs diabetic plus alagebrium

ALT, alagebrium

diabetic control animals (Fig. 4). Delayed intervention treatment with either alagebrium or quinapril failed to significantly affect *Cd36* gene expression. Treatment with alagebrium tended to attenuate *Ager* expression ($p=0.057$) (Table 2). Neither alagebrium nor quinapril treatment significantly attenuated RAGE protein levels (Fig. 4) when given as delayed intervention for 10 weeks.

Effect of delayed intervention with alagebrium or quinapril on vascular inflammation

Oxidative stress Expression of *Ncf1*, encoding NADPH oxidase subunits p47phox, and also of *Cybb*, encoding gp91phox, was upregulated in the diabetic vasculature (Table 2). Treatment with alagebrium did not affect *Cybb* expression. However, there was significant attenuation of *Ncf1* expression when compared with quinapril-treated animals, but not untreated diabetic animals. Nitrotyrosine, an index of peroxynitrite-mediated tissue damage, was significantly increased in the aortas of untreated diabetic *Apoe*$^{-/-}$ mice when compared with non-diabetic control animals (Fig. 5). Treatment with both alagebrium and quinapril significantly ameliorated nitrotyrosine staining, suggesting that delayed intervention with both therapies was associated with decreased oxidative stress in the vasculature. Despite the increased gene expression of *Cybb* observed with delayed intervention with quinapril, nitrotyrosine levels were reduced in the aorta, suggesting an overall reduction in oxidative stress with quinapril.

Inflammatory markers Diabetes was associated with an upregulation of the aortic gene expression for vascular cell adhesion molecule 1 (VCAM1), the pro-inflammatory marker and leucocyte adhesion molecule, as well as upregulation of expression of *Ccl2*, which encodes monocyte chemotactic protein 1 (MCP-1) (Table 2). Treatment with alagebrium did not significantly alter *Vcam1* or *Ccl2* expression; however, delayed intervention with quinapril significantly attenuated *Vcam1* expression in diabetic animals. Staining for the macrophage marker F4/80 was increased in aortas of untreated diabetic mice compared with non-diabetic control mice; however, neither treatment significantly reduced F4/80 levels (ESM Fig. 3). Expression of *Il1b*, which encodes IL-1β, a cytokine that increases adhesion factors on endothelial cells to enable transmigration of leucocytes, was highly upregulated in diabetic vascular tissue (Table 2). Treatment with both alagebrium and quinapril significantly reduced *Il1b* expression in aortas from diabetic animals.

Vascular collagen content as assessed by trichrome staining was significantly increased in the aorta of diabetic animals (Fig. 6). Delayed intervention therapy with alagebrium significantly attenuated this increase in vascular collagen deposition, whereas treatment with quinapril did not have this effect.

Renin–angiotensin system

Reflecting an activated vascular renin–angiotensin system (RAS), diabetic *Apoe*$^{-/-}$ mice had a marked increase in expression of the gene for angiotensin II type 1a receptor (AT1a; *Agtr1a*) and angiotensin II type 2 receptor (AT2; *Agtr2*). Treatment with both alagebrium and quinapril was associated with reduced expression of both these genes (Table 2). The expression of the gene encoding ACE was also upregulated by diabetes; however, neither treatment affected *Ace* expression.

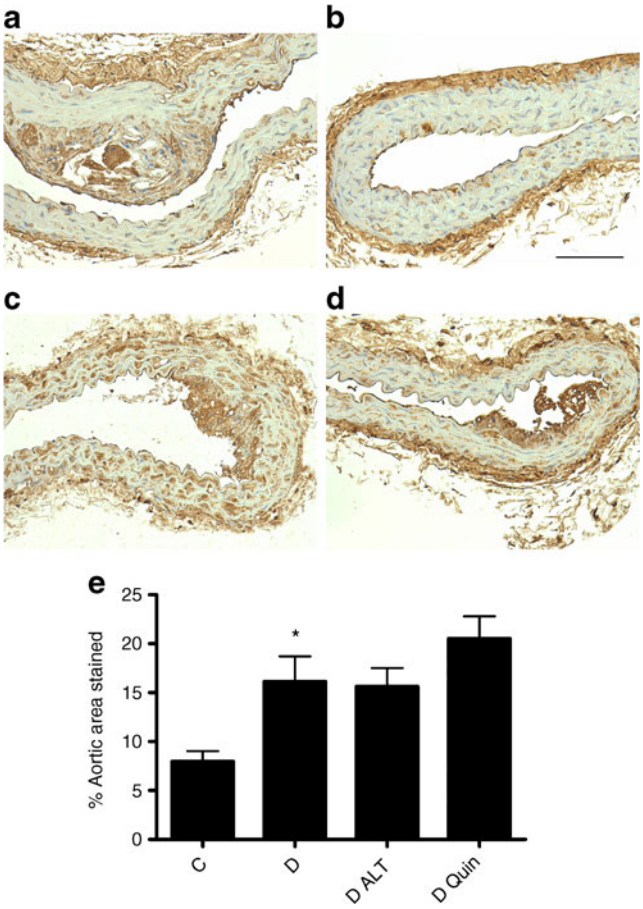

Fig. 4 **a**–**d** Photomicrographs of aorta from *Apoe*-knockout mice stained for RAGE: untreated diabetic mice (**a**); untreated non-diabetic control mice (**b**); diabetic mice + alagebrium, 1 mg/kg, for weeks 10–20 (**c**); diabetic mice + quinapril 30 mg/kg for weeks 10–20 (**d**). **e** Digital quantification of RAGE staining in the whole aorta (n=7–8 per group). Scale bar represents 100 μm. Data presented as means±SEM. *p<0.05 vs control animals. C, non-diabetic control mice; D, diabetic mice; D ALT, diabetic mice with delayed intervention with alagebrium, 1 mg/kg; D Quin, diabetic mice with delayed intervention with quinapril, 30 mg/kg

Discussion

Diabetes is associated with a significant increase in the size and complexity of the atherosclerotic plaque, contributing to increased cardiovascular events and premature mortality. Current treatment strategies attenuate, but do not prevent, cardiovascular complications and there remains an urgent need for new targets and interventions.

Prolonged hyperglycaemia, dyslipidaemia and oxidative stress in diabetes are associated with the production and accumulation of AGEs. AGEs are thought to directly contribute to the development and progression of cardiovascular disease in diabetes by promoting vascular dysfunction and injury, accelerating atherogenesis. As a result, novel therapeutic agents to reduce the accumulation of AGEs in diabetes have gained interest as potential cardioprotective approaches.

Clinically, it is more likely that diabetic patients will present with a certain degree of underlying atherosclerosis prior to the initiation of treatment. Indeed, cardiovascular disease is often silent in patients with diabetes. Most experimental studies, however, use a preventive treatment regimen, commencing therapies almost immediately after inducing diabetes. Studies using a delayed intervention protocol in established atherosclerosis are rare, with the exception of studies described for sRAGE [5].

Our group has previously demonstrated a significant reduction of atherosclerosis with alagebrium treatment in diabetic *Apoe*$^{-/-}$ mice when given as a preventive therapy,

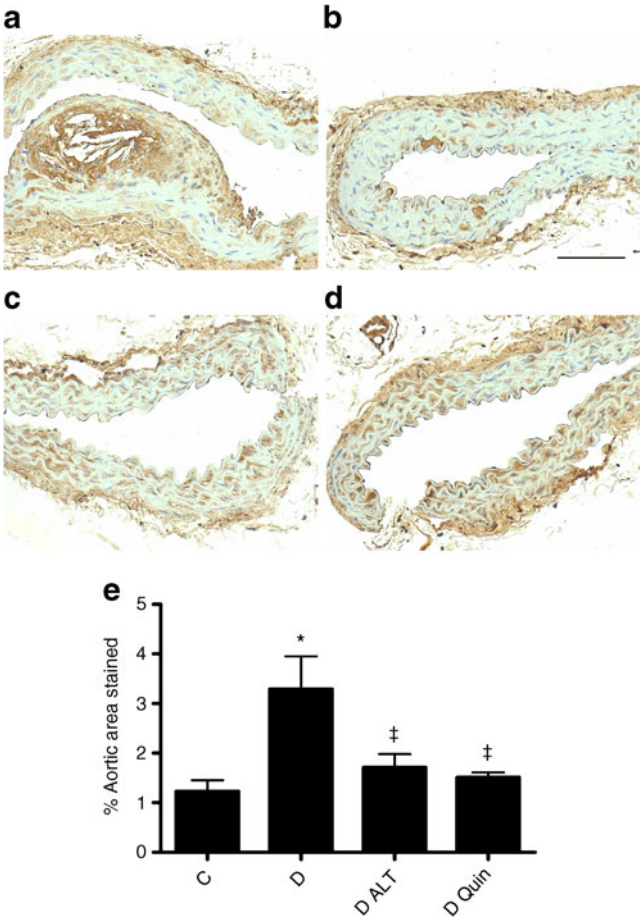

Fig. 5 **a**–**d** Photomicrographs of mouse aorta stained for a marker of oxidative stress, nitrotyrosine: untreated diabetic mice (**a**); untreated non-diabetic control mice (**b**); diabetic mice+alagebrium, 1 mg/kg, for weeks 10–20 (**c**); diabetic mice+quinapril 30 mg/kg for weeks 10–20 (**d**). **e** Digital quantification of nitrotyrosine staining in the whole aorta (n=5–8 per group). Scale bar represents 100 μm. *p<0.05 vs control mice; ‡p<0.05 vs diabetic mice. C, non-diabetic control mice; D, diabetic mice; D ALT, diabetic mice with delayed intervention with alagebrium, 1 mg/kg; D Quin, diabetic with delayed intervention with quinapril, 30 mg/kg

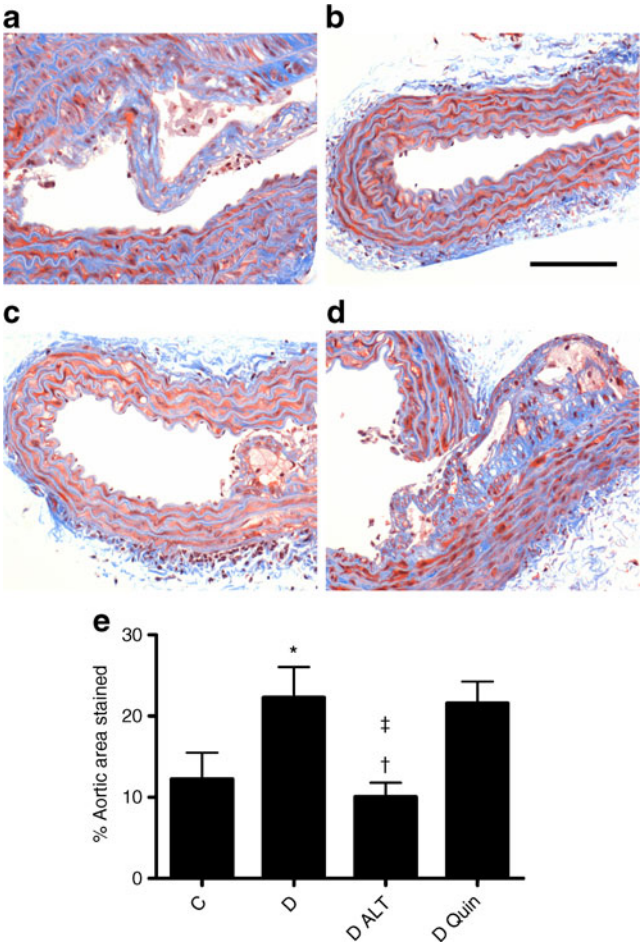

Fig. 6 a–d Photomicrographs of trichrome stained mouse aorta: untreated diabetic mice (**a**); untreated non-diabetic control mice (**b**); diabetic mice+alagebrium, 1 mg/kg, for weeks 10–20 (**c**); diabetic mice + quinapril 30 mg/kg for weeks 10–20 (**d**). **e** Digital quantification of collagen (blue) staining in the whole aorta (n=4–8 per group). Scale bar, 100 μm. *p<0.05 vs control mice; $^{\dagger}p$<0.05 vs diabetic mice with delayed intervention with quinapril; $^{\ddagger}p$<0.05 vs diabetic mice. C, non-diabetic control mice; D, diabetic mice; D ALT, diabetic mice with delayed intervention with alagebrium, 1 mg/kg; D Quin, diabetic mice with delayed intervention with quinapril, 30 mg/kg

directly after the induction of diabetes [8]. The current study investigated the effect of a delayed intervention strategy using two separate anti-AGE compounds, alagebrium and pyridoxamine, in a model where diabetes-associated atherosclerosis was already established. We also investigated the effects of the ACE inhibitor, quinapril, which has been shown to have anti-atherosclerotic effects when given as a preventive therapy in this animal model [12].

At the time of each intervention (commencing 10 weeks after the induction of diabetes), diabetic *Apoe*$^{-/-}$ mice have already accumulated plaque covering 5–10% of the total aortic surface area. In addition, the vessel wall already contains increased amounts of AGEs. Despite this, we have shown for the first time that delayed intervention treatment in diabetes with the anti-AGE agents alagebrium and pyridoxamine can attenuate atherosclerotic plaque formation in diabetic mice with established atherosclerosis. Furthermore, the anti-atherosclerotic effects of alagebrium were comparable with those observed with the ACE inhibitor quinapril. These anti-atherosclerotic effects using two distinct anti-AGE compounds suggest that even when given as a delayed intervention, anti-AGE therapies may present a new potential regimen for the attenuation of progression of diabetes-associated atherosclerosis.

The mechanism(s) by which alagebrium and pyridoxamine exert their anti-atherosclerotic effects in vivo remains to be fully established. While both interventions attenuated the further progression of established diabetes-associated atherosclerosis, none of the interventions regressed plaque development or associated markers of inflammation and fibrosis to levels less than those observed at 10 weeks of diabetes, the time-point when the treatment was started. Although both alagebrium and pyridoxamine have been reported to be able to cleave covalent cross-linked compounds in vitro [8, 13–15], we demonstrated that vascular AGE and RAGE protein levels were not reduced by the delayed treatment with alagebrium. Consequently, their anti-atherosclerotic actions in our model appear to be associated with inhibiting AGE formation, rather than breaking pre-formed AGE cross-links. Certainly, alagebrium was able to reduce the accumulation of circulating methylglyoxal and albumin fluorescence, a biomarker of AGE modification [16]. However, other vasculoprotective actions have also been attributed to alagebrium, including inhibition of RAGE-dependent protein kinase C signalling [17] and antioxidant actions. In our study, delayed intervention with alagebrium was associated with a significant attenuation of oxidative stress in the vascular wall, as measured by accumulation of nitrotyrosine residues. By contrast, it has previously been found that alagebrium, 10 mg kg^{-1} per day^{-1}, did not decrease renal nitrotyrosine staining in the kidney of diabetic rats when given as a delayed intervention [18].

RAS blockade with an ACE inhibitor or an angiotensin II receptor blocker is considered to be the first-line treatment in the prevention of diabetic end-organ complications [19, 20]. We have previously shown that the RAS plays an important role in the development of diabetes-associated atherosclerosis [21]. The current study is the first to demonstrate that delayed intervention therapy with quinapril attenuates the development of diabetes-associated atherosclerosis in established diabetes. This was accompanied by attenuation of *Agtr1a* and *Agtr2* expression in the diabetic aorta, as well as expression of genes for pro-inflammatory mediators such as VCAM1 and IL-1β. This supports the hypothesis that both the RAS and AGEs act to progress diabetes-associated atherosclerosis [4, 22].

Whilst the blood pressure-lowering effects of quinapril undoubtedly contributed to the anti-atherosclerotic effect of

this delayed therapy, previously our group have demonstrated that blood pressure reduction alone is not sufficient to account for the reduction of diabetes-associated atherosclerosis in this model [21]. Previously, we have found that this dose of quinapril reduced aortic atherosclerosis and nitrotyrosine staining in the diabetic *Apoe*$^{-/-}$ when used as a preventive (20 week) therapy [12]. The current study found that delayed intervention therapy with quinapril also decreased aortic nitrotyrosine levels. This, together with decreases in plasma AGEs in diabetic animals, suggests that quinapril exerts both anti-AGE and anti-oxidant effects in established atherosclerosis.

In summary, the present studies show that delayed intervention with two different anti-AGE compounds retarded but did not completely arrest the continued development of established diabetes-associated atherosclerosis. These effects were comparable with those achieved by ACE inhibition. This re-affirms the importance of ACE inhibitor treatment in established diabetes-associated atherosclerosis and provides evidence that novel treatments, such as those targeting AGEs, could be effective in combating established diabetic macrovascular disease. Although the clinical utility of AGE inhibition remains to be firmly established, these findings suggest that treatments that target the AGE–RAGE pathway may be effective in attenuating diabetes-associated atherosclerosis in patients with pre-existing macrovascular disease.

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