

Advanced Glycation End Product Interventions Reduce Diabetes-Accelerated Atherosclerosis

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Advanced glycation end product (AGE) formation may contribute to the progression of atherosclerosis, particularly in diabetes. The present study explored atherosclerosis in streptozotocin-induced diabetic apolipoprotein E-deficient (apoE^{-/-}) mice that were randomized ($n = 20$) to receive for 20 weeks no treatment, the AGE cross-link breaker ALT-711, or the inhibitor of AGE formation aminoguanidine (AG). A sixfold increase in plaque area with diabetes was attenuated by 30% with ALT-711 and by 40% in AG-treated mice. Regional distribution of plaque demonstrated no reduction in plaque area or complexity within the aortic arch with treatment, in contrast to the thoracic and abdominal aortas, where significant attenuation was seen. Diabetes-associated accumulation of AGEs in aortas and plasma and decreases in skin collagen solubility were ameliorated by both treatments, in addition to reductions in the vascular receptor for AGE. Collagen-associated reductions in the AGEs carboxymethyllysine and carboxyethyllysine were identified with both treatments. Diabetes was also accompanied by aortic accumulation of total collagen, specifically collagens I, III, and IV, as well as increases in the profibrotic cytokines transforming growth factor- β and connective tissue growth factor and in cellular α -smooth muscle actin. Attenuation of these changes was seen in both treated diabetic groups. ALT-711 and AG demonstrated the ability to reduce vascular AGE accumulation in addition to attenuating atherosclerosis in these diabetic mice. *Diabetes* 53:1813–1823, 2004

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AG, aminoguanidine; AGE, advanced glycation end product; ALE, advanced lipoxidation end product; apoE, apolipoprotein E; α -SMA, α -smooth muscle actin; CEL, Nε(carboxyethyl)lysine; CML, Nε(carboxymethyl)lysine; CTGF, connective tissue growth factor; RAGE, receptor for AGE; RAS, renin-angiotensin system; sRAGE, soluble RAGE; TGF- β , transforming growth factor- β .

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Macrovascular complications develop in >50% of the diabetic population and account for 50–60% of the mortality in this high-risk population (1). Dyslipidemia, hypertension, and hyperglycemia all play a role in the development of diabetes-associated atherosclerosis (2), although the specific contributions from each of these independent risk factors remains controversial.

The in vivo relevance of the Maillard reaction and the subsequent production of advanced glycation end products (AGEs) in the macrovascular complications of diabetes was first emphasized in studies using the inhibitor advanced glycation aminoguanidine (AG) (3). Subsequent studies that used exogenous administration of AGEs to mimic diabetic serum concentrations indicated that AGEs could induce atherosclerosis (4). Furthermore, AGEs interact with endothelial cells to induce the expression of atherogenic adhesion molecules implicated in atherogenesis (5,6).

More recently, the contribution of not only AGEs formed in the presence of both oxygen and sugar, namely glycoxidation products, but those derived from lipoxidation (7) or advanced lipoxidation end products (ALEs) to diabetic vascular complications has been suggested (8). Studies in experimental obese Zucker rats, a model of insulin resistance with the AGE formation inhibitor pyridoxamine, have shown marked attenuation of the characteristic hyperlipidemia, reduced ALEs, and hypertension, indicating interactions between AGEs and lipids (9). The role of these ALEs in atherosclerosis, a disease process linked not only to hyperglycemia but also to dyslipidemia is yet to be elucidated.

One of the seminal in vivo studies performed in experimental diabetes-associated atherosclerosis demonstrated attenuation of plaque formation with the soluble receptor for AGEs (sRAGE) (10). It should be noted that RAGE interacts with a range of other molecules in addition to AGEs, such as amphotericin and S-100 (11), which could themselves participate in the atherosclerotic process. Therefore, it remains unresolved as to whether the anti-atherosclerotic effects of sRAGE are primarily related to its effects in inhibiting AGE-mediated events or via other mechanisms, although the former remains the most likely.

AG is the most extensively studied of the AGE formation inhibitors and has previously been shown to attenuate vascular complications (3). More recently, another thera-

peutic approach has involved the use of a thiazolium derivative ALT-711, a compound that is suggested to be capable of breaking AGE cross-links, thereby removing preformed AGEs (12). ALT-711 has proved successful as an intervention therapy in established diabetic microvascular complications (13,14) and has also been reported to improve vascular compliance in aging humans (15). Other studies in the diabetic context have shown that ALT-711 attenuates both cardiac (14) and renal (13) accumulation of AGEs in association with benefits on renal and cardiac injury. Therefore, the present study examined the effects of ALT-711 and compared it with the more traditional AGE formation inhibitor AG in a well-characterized model of diabetes-associated atherosclerosis (10,16), the streptozotocin-induced diabetic apolipoprotein E-deficient (apoE^{-/-}) mouse. Plaque area, complexity, and fibrosis in the aorta were examined in the presence and absence of these treatments. Collagen solubility and expression were also assessed with emphasis placed on both fibrillar and membranous collagens. Finally, AGE and ALE accumulation and expression of RAGE were evaluated.

RESEARCH DESIGN AND METHODS

Experimental model. Six-week-old homozygous apoE-deficient male mice (backcrossed 20 times from C57BL/6 background; Animal Resource Centre, Canning Vale, WA, Australia) were studied in accordance with guidelines provided by the Alfred and Baker Animal Ethics Committee. Mice were rendered diabetic by daily injection of streptozotocin at a dosage of 55 mg/kg for 5 consecutive days. Control animals were given daily injections of vehicle (citrate buffer) alone. Groups of diabetic animals ($n = 20/\text{group}$) were then randomized after 1 week of recovery to receive 1) no treatment; 2) AGE cross-link breaker ALT-711 [4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride; Alteon, Ramsey, NJ], gavaged at $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (DALT); or 3) the AGE formation inhibitor AG hydrocarbonate (Fluka Pharmaceuticals) at $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (DAG; 1 g/l in drinking water). All groups of animals were followed for 20 weeks. Mice were allowed access to standard mouse diet and water ad libitum. An initial pilot study using two doses of ALT-711 (gavaged at 10 or $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) revealed no differences between the two doses in terms of the primary end point of plaque accumulation or aortic AGE accumulation. Therefore, the definitive study involving comparison with AG was performed using $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ALT-711.

At the conclusion of the study, glycated Hb (GHb) was measured by high-performance liquid chromatography (17). Total cholesterol, HDL, and triglyceride concentrations were measured by autoanalyzer. LDL cholesterol was calculated by the Friedewald formula (18). Systolic blood pressure was assessed by a computerized, noninvasive tail-cuff technique in conscious mice (19).

After 20 weeks, after anesthesia with intraperitoneal pentobarbital sodium (60 mg/kg body wt; Nembutal, Boehringer Ingelheim, Mannheim, Germany), aortas were rapidly dissected and snap-frozen in liquid nitrogen. A subset of aortas from each group ($n = 10$ mice per group) were removed and fixed in neutral-buffered formalin for subsequent evaluation of lesions.

Evaluation of atherosclerotic lesion area. Two approaches were used to evaluate the atherosclerotic lesions as described previously (16). First, macroscopic assessment of en face whole aorta segments was performed. The entire aorta was cleaned of fat, opened longitudinally, and dissected into three segments: aortic arch, descending thoracic aorta, and abdominal aorta. After staining with Sudan IV-Herxheimer's solution (Sigma Chemical Co, Carpinteria, CA), aortas were pinned out flat, and images were digitized on a dissecting microscope (Olympus SZX9; Olympus Optics, Tokyo, Japan) equipped with a high-resolution camera (Zeiss Axiocam; Zeiss Instruments, Göttingen, Germany). The digitized images were evaluated, and the lesion area was evaluated by calculation of the proportion of aortic intimal surface occupied by red stain in each of the three aortic segments using Image SXM program software (National Institutes of Health, Bethesda, MD).

The aortic segments studied above were then embedded in paraffin on end and cross-sectioned at $4 \mu\text{m}$ for subsequent immunohistochemistry, *in situ* hybridization, and histological evaluation. Secondary histological assessment of lesions was performed on sections stained with Masson's Trichrome as described previously (16). Total collagen was quantified by calculation of the area of aniline blue staining occupied within the plaques by Optimas 6.2

Software (Optimas 6.2; Video Pro-32, Bedford Park, SA, Australia) associated with a JVC video camera and Olympus microscope.

Plasma AGE peptide analysis. Fluorescence was assayed in duplicate 20- μl plasma aliquots as described previously (13). Characteristic fluorescence (excitation 370 nm, emission 440 nm) has been previously attributed to the accumulation of AGE cross-links (20,21). Briefly, samples were deproteinated and delipidated by addition of 0.15 mol/l trichloroacetic acid and chloroform (22), followed by centrifugation and removal of the upper aqueous phase. Fluorescence of the supernatant was determined (excitation 370 nm, emission 440 nm) using an on-line high-performance liquid chromatography injector (Waters, Milford, MA) and expressed per arbitrary protein unit at 280 nm.

Isolation of skin collagen and analysis of AGEs/ALEs. Mouse skin collagen was prepared as described previously for rat skin collagen (8). In brief, insoluble collagen was isolated from 1.5-cm pieces of skin after removal of fat and hair with a razor blade and subsequent sequential extractions with 1.0 mol/l NaCl, 0.5 mol/l acetic acid, and delipidation with chloroform:methanol (1:2). The collagen was then lyophilized and stored at -20°C until analyzed for AGE/ALE content. The AGEs/ALEs N ϵ (carboxymethyl)lysine (CML) and N ϵ (carboxyethyl)lysine (CEL) were quantified by isotope dilution and selected ion monitoring gas chromatography-mass spectrometry (23) and normalized to their parent amino acid lysine.

Evaluation of cross-linking. Skin collagen (3 mg), prepared as above, was used for cross-linking analysis and added to 7.5 ml of 0.5 mmol/l acetic acid containing $10 \mu\text{mol/l}$ pepsin (Sigma Chemical Co.) and incubated at 37°C with gentle agitation (60 Hz). Aliquots of 250 μl were removed at 0, 4, 8, 12, 16, and 24 h of digestion. The conditions were chosen on the basis of preliminary studies that showed significant solubilization of collagen from nondiabetic animals at 1 h and maximum solubilization (>98%) after 24 h. The collagen content of these samples was determined after acid hydrolysis (6 mol/l HCl for 24 h at 110°C) using the procedure of Stegemann and Stadler (24). Collagen solubility was expressed as a function of time with the 0 time point providing 0% digestion and the 24-h time point providing 100% digestion. The collagen content at each time point of pepsin digestion was expressed as a percentage of the total recoverable collagen after 24 h. The time taken for digestion of 50% of the collagen was determined for each of the groups using derivatization of area under the curve.

Skin fluorescence (at excitation 370 nm, emission 440 nm) was determined in 24-h acid-hydrolyzed collagen preparations in the presence of 0.1 mol/l NaBH₄ via a flow injection system adapted from Wrobel et al. (22) using a Waters 470 spectrophotometer/fluorometer (Waters). For correcting for differences in sample collagen content, fluorescence intensity was expressed as arbitrary units of fluorescence per milligram of collagen.

Immunohistochemistry. A modification of the ABC Ig enzyme bridge technique (25) was used for immunohistochemistry. Formalin-fixed paraffin sections of aortas from previous en face studies were dewaxed and hydrated. Antigen retrieval by microwaving in citrate buffer (1.8 mmol/l citric acid, 8.2 mmol/l trisodium citrate) twice for 5 min was performed for connective tissue growth factor (1:500; rabbit anti-rCTGF; gift of Dr. S. Twigg, Department of Medicine, University of Sydney) (26) and transforming growth factor- β (TGF- β ; 1:250; Santa Cruz Biotechnology, Santa Cruz, CA). Slides for collagen immunostaining were porcine pepsin digested (0.4%) in 0.5% nonfat milk powder diluted in Tris-buffered saline containing 0.05% Tween 20. After incubation with 0.3% hydrogen peroxide for 20 min, sections were incubated with protein-blocking agent (Lipshaw, Pittsburgh, PA) for 20 min and then incubated with primary antibody overnight at 4°C . CML is the major epitope recognized by the monoclonal anti-AGE antibody, 4G9, used in this protocol (1:500, Alteon) (27). Other primary antibodies used were polyclonal goat anti-RAGE (1:250; Chemicon, Temecula, CA); goat anti-human collagen I, III, and IV (1:80, 1:75, and 1:800, respectively; Southern Biotechnologies, Birmingham, AL); and mouse anti-human α -smooth muscle actin (α -SMA; 1:50; Dako).

Tissue sections were stained consecutively with biotinylated IgG for 10 min and avidin-biotin horseradish peroxidase complex for 15 min (Vectastain ABC ELITE kit; Vector Laboratories, Burlingame, CA) before a substrate solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) was added. Sections were counterstained in Harris' hematoxylin and mounted in dePex (BDH; Merck, Poole, U.K.). Negative control sections had the omission of the primary antibody. Positive control tissues were also included. Quantification of aortic cross-section immunostaining was completed by computer-aided densitometry (Optimas 6.2; Video Pro-32), whereby a total of 10 slides that contained at least 6 sections ($\times 100$) per group were counted. Results were expressed as proportional area of positive staining (28) in cross-sections.

In situ hybridization. Localization of CTGF mRNA was determined by *in situ* hybridization as previously described, with the inclusion of a sense riboprobe to determine background hybridization (16). Briefly, antisense CTGF and sense riboprobes labeled with ^{35}S CTP were prepared using the Promega Transcription System (Promega, Madison, WI). Four-micron sec-

TABLE 1
Metabolic parameters for all groups at week 20

Parameters	Control	Diabetes	Diabetes + ALT-711	Diabetes + AG
<i>n</i>	20	20	20	20
GHb (%)	3.8 ± 0.1	13.6 ± 0.4*	14.1 ± 1.2*	11.9 ± 0.5*†
Body weight (g)	30.0 ± 0.5	21.1 ± 0.3*	22.0 ± 1.0*	21.0 ± 0.3*
Systolic blood pressure (mmHg)	117 ± 2	118 ± 5	104 ± 4§	125 ± 6
Total cholesterol (mmol/l)	14.2 ± 0.5	35.6 ± 2.4*	22.0 ± 3.6*§	31.6 ± 1.2*‡
HDL cholesterol (mmol/l)	4.5 ± 0.2	8.2 ± 0.7*	7.5 ± 1.1*	6.5 ± 0.3*§
LDL cholesterol (mmol/l)	9.2 ± 0.3	26.1 ± 1.5*	13.9 ± 2.4§	24.1 ± 0.7*‡
Triglycerides (mmol/l)	0.9 ± 0.1	1.9 ± 0.3*	1.2 ± 0.3†	2.0 ± 0.3*‡

Data are means ± SE except for GHb and body weight, which are means ± SD. **P* < 0.001 vs. control group; †*P* < 0.05 vs. diabetic group; ‡*P* < 0.001 vs. diabetes + ALT group; §*P* < 0.001 vs. diabetic group.

tions were incubated in 125 µg/ml Pronase E at 37°C, postfixed in 4% paraformaldehyde, and air dried. The slides were then incubated overnight at 60°C in hybridization buffer that contained 2 × 10⁴ cpm/µl ³⁵S-labeled riboprobe, 0.72 mg/ml yeast RNA, 50% deionized formamide, 100 mmol/l dithiothreitol, 10% dextran sulfate, 0.3 mol/l NaCl, 10 mmol/l Na₂HPO₄ (pH 7.5), 5 mmol/l EDTA (pH 8.0), 0.02% BSA, 0.02% Ficoll 400, and 0.02% polyvinylpyrrolidone. Coverslips were removed, and sections were rinsed and then incubated at 37°C in 150 µg/ml RNase A for 1 h. Samples were washed, air dried, and exposed to BioMax MR film for 3–5 days. For microscopic localization, slides were coated in Amersham LM-1 emulsion, dried for 1 h, and incubated at 4°C in a light-proof container with desiccant for a period of 2–4 weeks according to the autoradiography results and developed photographically as previously described.

Statistical analysis. Results are expressed as means ± SE unless otherwise specified. Analyses were performed by ANOVA followed by post hoc analysis using Fischer's least significant difference method, correcting for multiple comparisons (Statview V). *P* < 0.05 was considered to be statistically significant.

RESULTS

Metabolic parameters. Plasma glucose and GHb were increased in all diabetic animals as compared with the control group (Table 1). There was a modest decrease in GHb in AG-treated animals. Diabetic mice also had decreases in body weight, which were not affected by either treatment (Table 1). Systolic blood pressure remained unchanged between control and diabetic mice, although there was a significant reduction in blood pressure noted in the DALT treatment group (Table 1). When compared with untreated diabetic mice, those that were treated with ALT-711 showed significant reductions in plasma total cholesterol, LDL cholesterol, and triglycerides. AG treatment did not affect plasma triglycerides and had a modest effect on HDL cholesterol, but no statistical changes were seen with respect to total or calculated LDL cholesterol (Table 1).

Atherosclerotic plaque Areas. En face analysis of total aortic plaque area revealed an approximately sixfold increase in the untreated diabetic as compared with control mice (Fig. 1A). The lesions throughout the aortas of these diabetic animals appeared complex and fibrous as compared with the control apoE^{−/−} animals, which seemed to have mostly fatty streaks with complex plaques evident only in the aortic arch. Both AG- and DALT-treated animals demonstrated significant abrogation in total plaque area and in the complexity of plaques. There was no attenuation of plaque area seen in any treatment group in the aortic arch region (Fig. 1B), and these lesions appeared fibrous and complex in all groups. By contrast, in the thoracic and abdominal regions, complex plaques were identified primarily in the untreated diabetic group.

Each of the treatments administered to diabetic animals afforded a similar degree of attenuation in plaque lesion area in both the thoracic (Fig. 1C) and abdominal (Fig. 1D) regions of the aorta.

Plasma content and aortic expression of AGEs and RAGE. Diabetes induced a modest but significant increase in plasma AGE peptides (Fig. 2A). These diabetes-induced increases were completely prevented by each of the treatments.

Immunohistochemical analysis of the nonfluorescent AGE CML in aortic cross-sections revealed significant increases in the untreated diabetic group (Fig. 2B and 3A). There was extensive CML staining localized within the vascular wall, particularly in smooth muscle cells as well as in the endothelial and adventitial layers (Fig. 3B). There was also CML staining within the cellular portion of diabetic plaques. The elastic laminae, however, remained unstained. The most effective attenuation of this vascular wall staining of CML was seen in the DALT groups (Fig. 3C), although the AG-treated group also showed significant reductions in staining for CML (Fig. 3D). It is interesting that there was still some CML immunostaining identified within plaques of the DALT group.

Quantification of RAGE expression by immunohistochemistry identified a significant increase in this receptor in untreated diabetic mice as compared with control apoE^{−/−} mice (Fig. 2C). RAGE was localized throughout the aortic wall, particularly within the endothelium and adventitia, although there was also staining evident within the plaques of the untreated diabetic group (Fig. 3E and F). Each of the treatments prevented the diabetes-induced expression of RAGE within the aorta (Figs. 2C and 3G and H). No immunostaining was evident in the negative control sections in which the primary antibody was omitted.

Skin collagen cross-linking and AGE/ALE analysis. Skin collagen from diabetic animals was less soluble than collagen taken from control animals. The time taken to reach 50% digestion of the diabetic collagen with pepsin was 8.4 h as compared with 3.8 h in control animals (Fig. 4A). Each of the apoE^{−/−} diabetic groups, which received treatment, demonstrated increased solubility of their skin collagen. The DALT and DAG groups showed similar findings with 50% digestion achieved at ~5 h in both groups.

Skin collagen-associated CML was increased by diabetes and was attenuated by both treatments, although AG seemed to be more effective (Fig. 4B). There was no

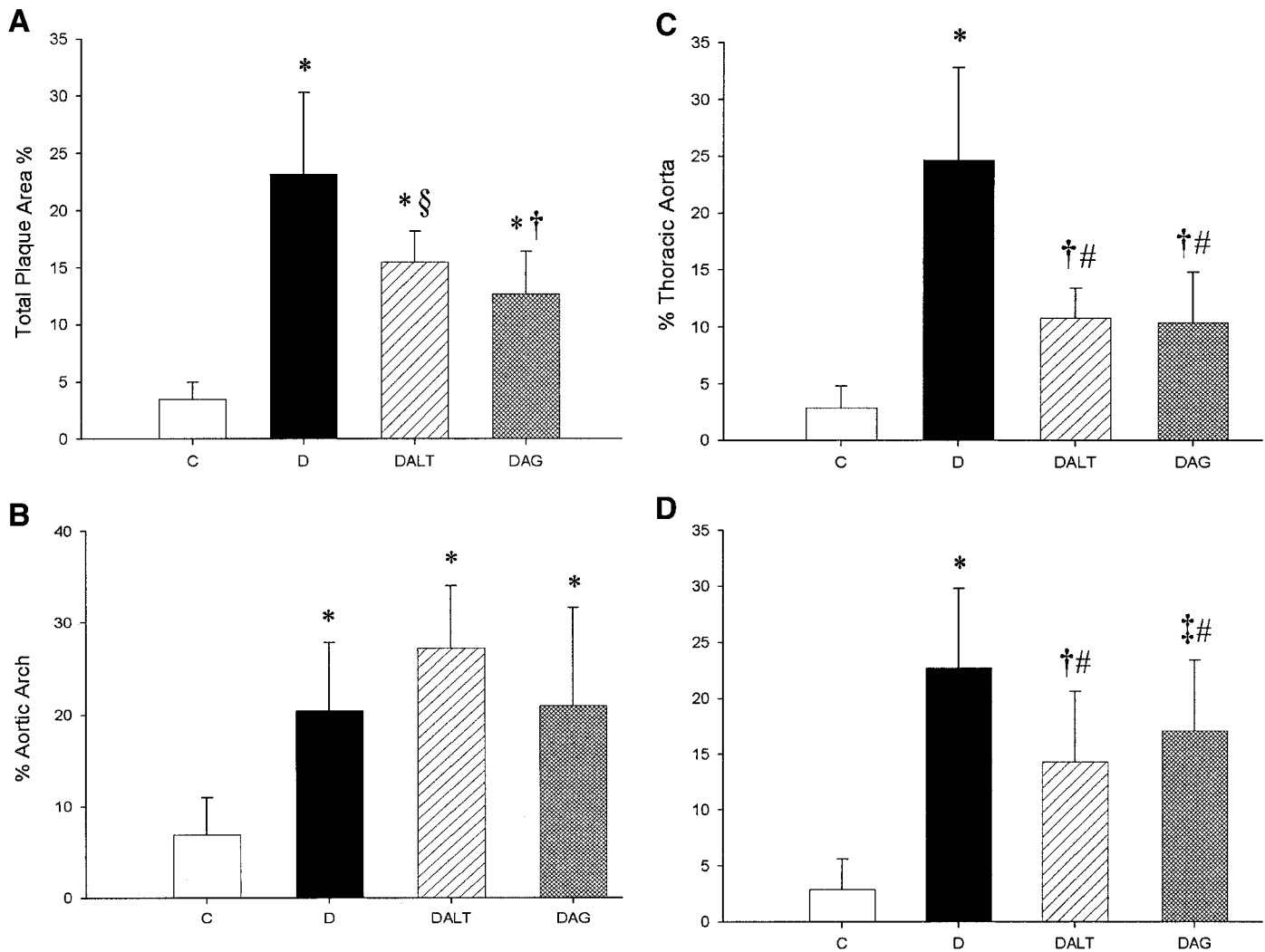


FIG. 1. Morphometric quantification of atherosclerotic plaque area in en face dissections of aortic segments (% aortic area; mean \pm SD). **A:** Total aorta. **B:** Aortic arch. **C:** Thoracic aorta. **D:** Abdominal aorta. * $P < 0.001$ vs. control group; † $P < 0.001$ vs. diabetic group; ‡ $P < 0.001$ vs. DALT group; § $P < 0.05$ vs. diabetic group; # $P < 0.05$ vs. control group.

increase in the AGE/ALE CEL, although AG did reduce collagen CEL to below control levels (Fig. 4C). Finally, untreated diabetic skin collagen showed increased AGE fluorescence (excitation 370 nm, emission 440 nm) as compared with control mouse skin collagen (Fig. 4D). Treatment of diabetic groups with ALT-711 or AG prevented the increase in skin collagen fluorescence.

Atherosclerotic lesion collagen content. The total collagen content in lesions was >10 times greater in the untreated diabetic aortic lesions as compared with nondiabetic controls (Table 2). This was related to significant increases in each of the collagens studied, specifically collagens I, III, and IV (Table 2). Treatment with ALT-711 attenuated the overall increase in plaque collagen content by ~50% but displayed differential effects on the three types of collagen studied. The reduction with ALT-711 treatment was most profound for collagen IV, which was normalized to nondiabetic levels, with collagen III attenuated by ~50%, whereas there was no effect on the increase in type I collagen. AG completely abrogated the diabetes-induced increase in total collagen, which was related in part to reductions in each of the collagen subtypes (Table 2).

Collagen I was identified throughout the aortic wall and within plaques of the diabetic apoE $^{-/-}$ mice. ALT-711 seemed to reduce the collagen content of the wall only, in contrast to AG, which provided no reduction in either plaque or aortic wall content of collagen I. Collagen III was localized to the outer layer of the elastic laminae throughout the vessel wall of diabetic animals. There was also staining perpendicular to the elastic laminae transversing the aortic media. In addition, collagen III was evident in the fibrous cap of complex plaques. Neither ALT-711 nor AG reduced the collagen III content immediately over plaques, although there was significant attenuation within the vessel wall in each of the treatment groups. Collagen IV was identified throughout the internal elastic laminae, particularly toward the outer surface. These layers were thickened in the diabetic apoE $^{-/-}$ mice with collagen IV deposition also evident within plaques. Both treatment groups showed a reduction in the content of collagen IV within the vessel wall and plaques, although the superficial surface staining of plaques remained unchanged.

Profibrotic cytokines. Aortic CTGF protein expression was induced by diabetes (Fig. 5A). CTGF mRNA and protein were localized within aortic plaques and in the

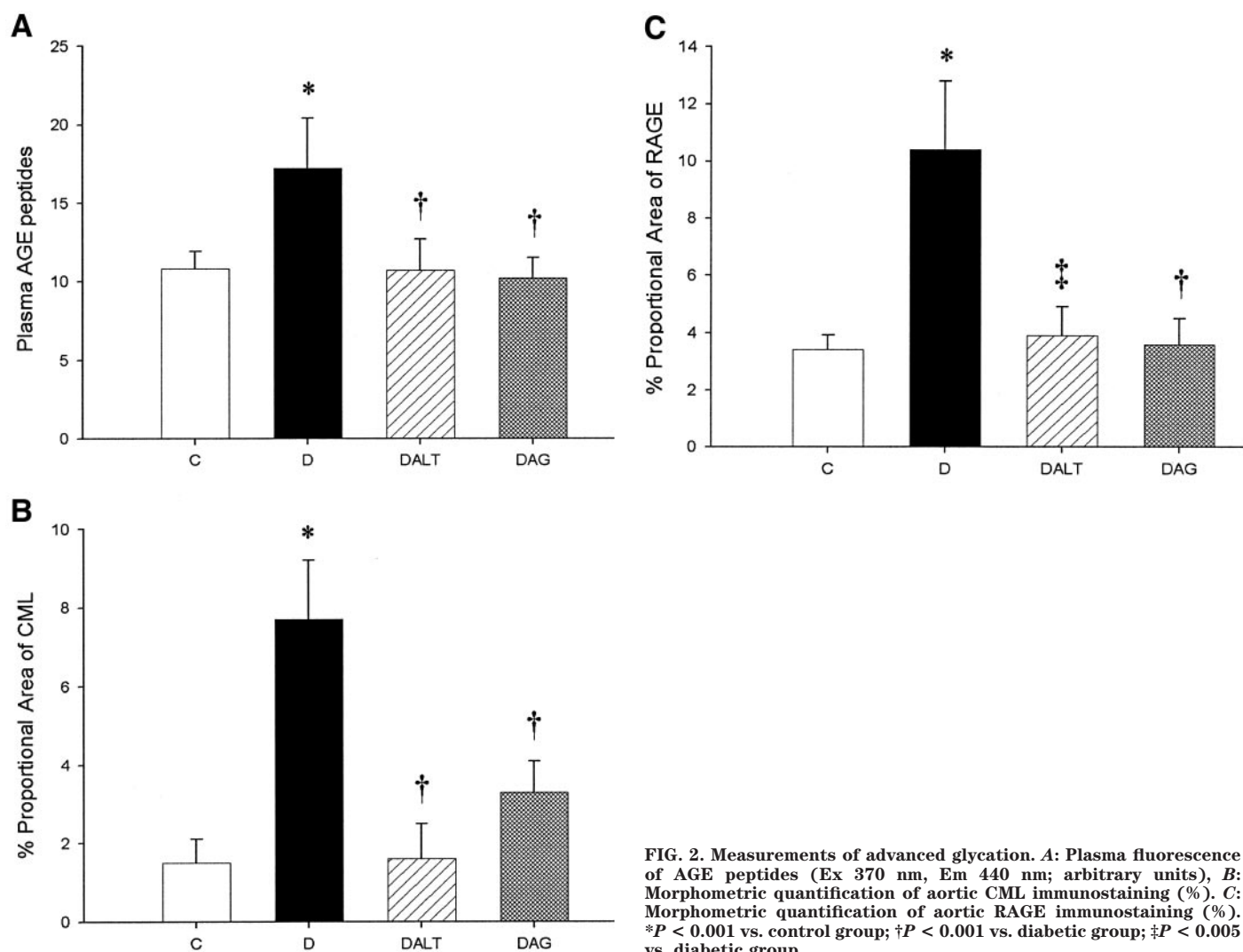


FIG. 2. Measurements of advanced glycation. **A:** Plasma fluorescence of AGE peptides (Ex 370 nm, Em 440 nm; arbitrary units), **B:** Morphometric quantification of aortic CML immunostaining (%). **C:** Morphometric quantification of aortic RAGE immunostaining (%). * $P < 0.001$ vs. control group; † $P < 0.001$ vs. diabetic group; ‡ $P < 0.005$ vs. diabetic group.

media of diabetic mice (Fig. 6). Both treatments attenuated these increases in CTGF gene and protein expression to levels seen in control apoE $^{-/-}$ mice. A similar pattern was seen for TGF- β 1 expression, which was increased in diabetic apoE $^{-/-}$ aortas as compared with control mice and was reduced in the treated groups (Fig. 5B).

α -SMA. α -SMA protein expression, as assessed by immunohistochemistry, was increased in the aortas from untreated diabetic as compared with control mice (Fig. 5C). This parameter was reduced by each of the treatments, although not to control levels (Fig. 5C).

DISCUSSION

The present study has clearly demonstrated that two disparate approaches to prevent aortic AGE accumulation—with either an inhibitor of AGE formation, AG, or an AGE cross-link breaker, ALT-711—are associated with attenuation of plaque area in a model of diabetes-associated atherosclerosis. These effects occurred in the context of reduced accumulation of AGEs within the aortas; reduced expression of RAGE, which has been implicated in the mediation of many of the biological effects of AGEs; alterations in both AGEs and ALEs present on skin collagen; and a reduction in the expression of pro-sclerotic growth factors and extracellular collagenous proteins.

AGE accumulation in aortic atheroma has been previously identified in nondiabetic patients (29). The present study confirms that AGEs are increased in diabetic apoE $^{-/-}$ mice as reported by Park et al. (10). Increases in aortic CML were found in association with increased CML in skin collagen. Analysis of skin collagen AGEs provides information about historical glycemic control as evidenced in type 1 diabetic patients in the Diabetes Control and Complications Trial (30) and significantly correlates with the long-term macrovascular outcomes of these patients recently published for the Epidemiology of Diabetes Interventions and Complications study (31), a follow-up trial of Diabetes Control and Complications Trial subjects. Recently, CML has been recognized as an ALE as well as an AGE because it can be formed during either lipid or carbohydrate oxidation (32). Thus, it may be that the increased CML in the atherosclerotic plaques and walls of the aortas observed in the present study is the result of both glycoxidation and peroxidation of lipoproteins and lipids. It is interesting that in the context of aortic plaque reduction, the ALT-711 treatment group had a reduction in plasma LDL cholesterol but lipid levels remained elevated. Our data provide the first report of the effects of ALT-711 on serum lipids, particularly in the apoE $^{-/-}$ mouse. Although in human trials, AG has demonstrated lipid-

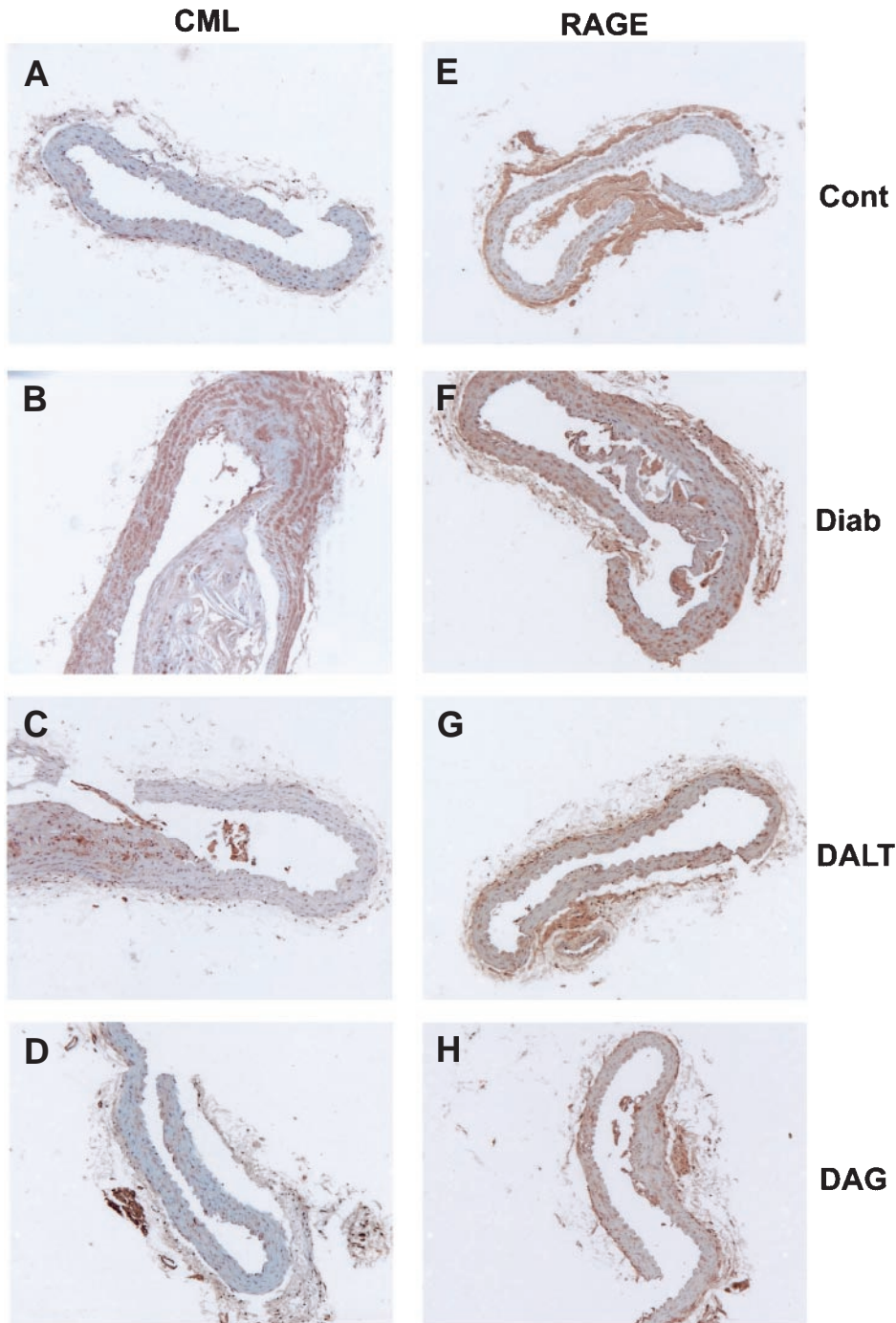


FIG. 3. Representative aortic immunostaining of CML for control (A), diabetic (B), DALT (C), and DAG (D) and aortic RAGE expression in control (Cont; E), diabetic (Diab; F), DALT (G), and DAG (H). Magnification $\times 200$.

lowering effects, previous data from other groups have suggested that in rodents, AG has no or minimal effect on plasma cholesterol and a modest effect on triglycerides (8). This is consistent with the present study in which no major effect of AG was demonstrated on the lipid profile in the diabetic apoE $^{-/-}$ mice. Importantly, despite different effects on lipids, both ALT-711 and AG were equally effective in reducing aortic CML content. The relative role of these agents on glycoxidation and lipoxidation in mediating their antiatherosclerotic effects remains to be determined. These pathways need to be considered further, however, with the recent demonstration that other agents that inhibit AGE accumulation may also influence lipid- and glucose-dependent pathways (8).

An important finding in the present study was the regional efficacy of the AGE inhibitors in attenuating plaque formation. Whereas both ALT-711 and AG had no significant effects on atherosclerosis within the aortic arch, previous studies by our own group using the same model have shown that angiotensin-converting enzyme (ACE) inhibition was effective in the aortic arch as well as in the thoracic and abdominal regions of the aorta (16). This suggests that the renin-angiotensin system (RAS), specifically the local RAS rather than systemic blood pressure, may be particularly important in plaque accumulation within this region of the aorta, especially in the context of a reduction in blood pressure by ALT-711, without effects on plaque formation at this site. The effects

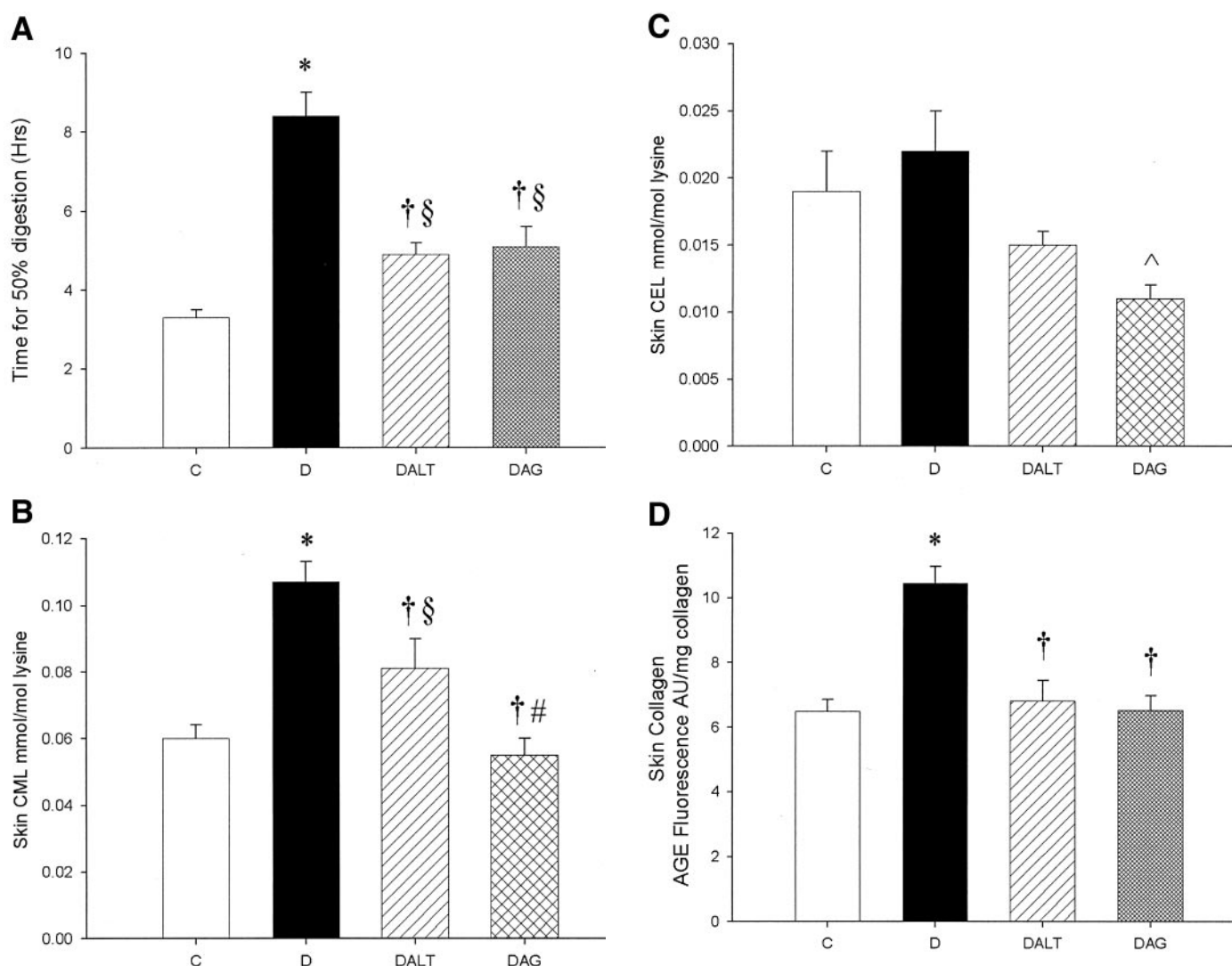


FIG. 4. Analysis of skin collagen. **A:** Time taken for 50% digestion with pepsin (area under the curve). **B:** Skin collagen CML content. **C:** Skin collagen CEL content. **D:** Skin collagen fluorescence (Ex 370 nm, Em 440 nm; arbitrary units/mg hydroxyproline). * $P < 0.001$ vs. control group; † $P < 0.001$ vs. diabetic group; ‡ $P < 0.001$ vs. DALT group; § $P < 0.01$ vs. control group; # $P < 0.05$ DALT vs. DAG group; ^ $P < 0.05$ vs. diabetic group.

of ALT-711 and AG on plaque accumulation in the thoracic and abdominal aorta were similar to those seen with perindopril. This suggests that metabolic pathways such as AGE accumulation predominate at these sites. Furthermore, with the recent demonstration both in vitro and in vivo that ACE inhibitors can act as potent inhibitors of AGE formation (33,34), it is possible that the effects of perindopril at these sites may be partly related to its ability to reduce AGE levels.

A range of studies from other groups have clearly defined that agents that interrupt AGE pathways influence the vascular tree. Administration of ALT-711 to aging patients abrogated vascular dysfunction attributed to vessel wall stiffening, in the absence of blood pressure reduction (15). AG has also been shown to have beneficial effects on vessel elasticity and endothelial dysfunction (3). Because AG is also an inhibitor of inducible nitric oxide synthase (35), it is possible that some of the beneficial

TABLE 2
Aortic lesion collagen content at week 20

Parameters	Control	Diabetes	Diabetes + ALT-711	Diabetes + AG
<i>n</i>	20	20	20	20
Total aortic lesion collagen (%)	3.9 ± 1.5	41.3 ± 3.7*	19.5 ± 3.5†‡	7.1 ± 1.3†§
Aortic collagen I (%)	2.2 ± 0.8	4.5 ± 0.8	3.9 ± 0.5	4.1 ± 0.9
Aortic collagen III (%)	0.9 ± 0.3	5.6 ± 0.6*	2.1 ± 0.3†	1.3 ± 0.2†
Aortic collagen IV (%)	2.7 ± 0.3	5.8 ± 0.8*	2.7 ± 0.5†	2.4 ± 0.3†

* $P < 0.001$ vs. control group; † $P < 0.001$ vs. diabetes group; ‡ $P < 0.005$ vs. control group; § $P < 0.05$ vs. diabetes + ALT group; || $P < 0.05$ vs. control group.

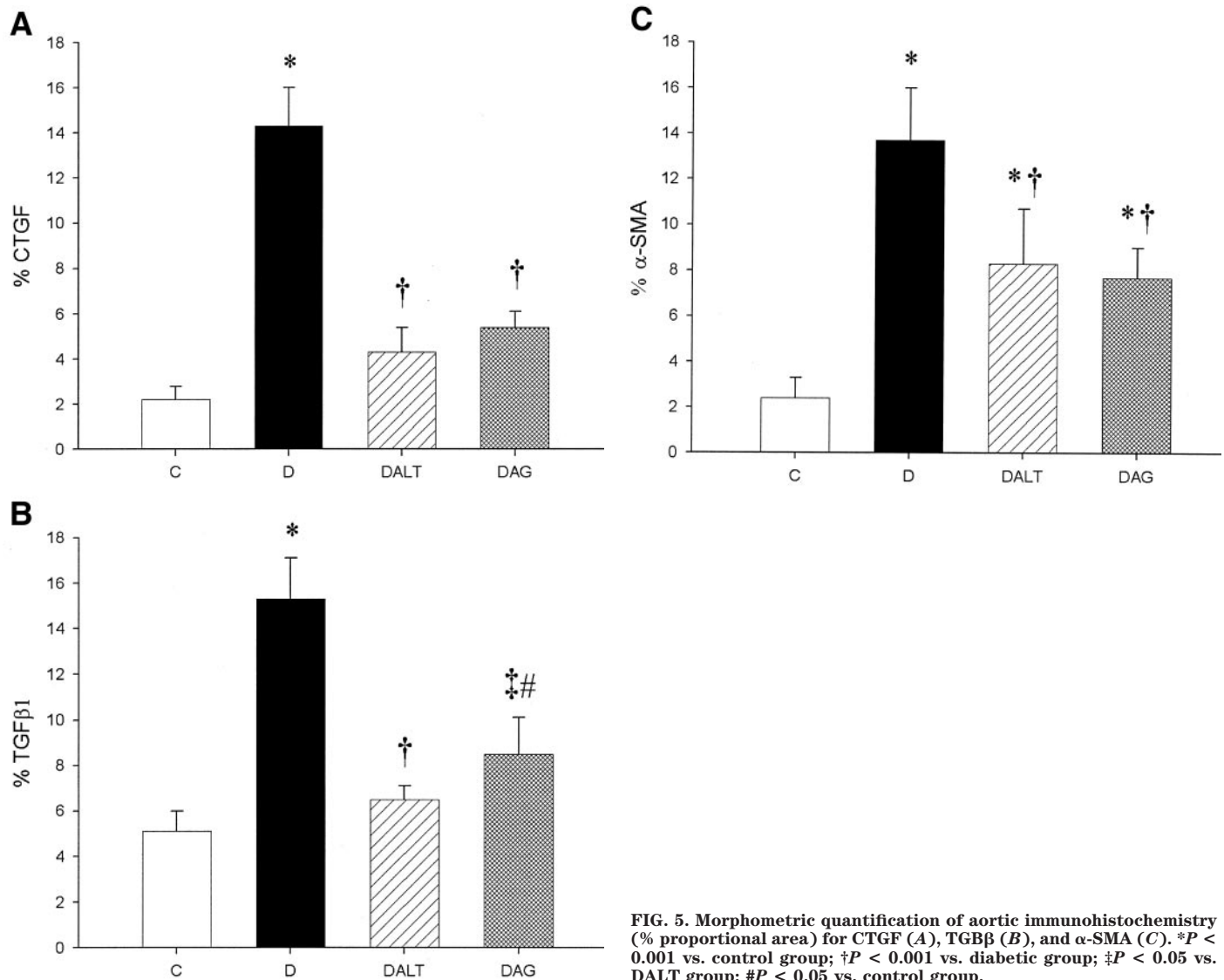


FIG. 5. Morphometric quantification of aortic immunohistochemistry (% proportional area) for CTGF (A), TGFβ (B), and α-SMA (C). * $P < 0.001$ vs. control group; † $P < 0.001$ vs. diabetic group; ‡ $P < 0.05$ vs. DALT group; # $P < 0.05$ vs. control group.

effects of this drug may be via this pathway. Indeed, genetic deficiencies in inducible nitric oxide synthase have been shown to be protective against the development of atheroma (36). Nevertheless, the ability of ALT-711 to attenuate atherosclerotic plaque formation in the present study and previous experiments documenting the efficacy of sRAGE in this model (37) emphasize that the predominant antiatherosclerotic effect of AG is likely to be mediated via its actions as an inhibitor of AGE formation.

ALT-711 and the related compound *N*-phenacylthiazolium bromide (38) have been reported by investigators to be AGE cross-link breakers capable of promoting the cleavage and removal of preformed AGEs (39). Indeed, although previously demonstrated to be an AGE cross-link breaker (12), ALT-711 has also been shown to reduce the accumulation of CML (13), and this effect has been postulated to be due to chelation of copper ions, thereby reducing copper catalyzed glycoxidation (40,41). One must be cautious about the interpretation of these *in vitro* data as these authors also did not find an effect of ALT-711 in contrast to studies defining its ability to cleave preformed AGEs (12). Skin collagen from the diabetic apoE^{-/-} mice was heavily cross-linked as assessed by a reduction in its

solubility in pepsin and an increase in fluorescent AGE content. Treatment of diabetic apoE^{-/-} mice with ALT-711 or AG reversed this defect in solubility and reduced the accumulation of AGEs. It is likely that the mechanism of action of these two compounds in conferring these effects differs. Although ALT-711 restores collagen solubility and reduces aortic and skin AGE accumulation, there was only partial attenuation of the diabetes-induced increase in aortic collagen content in this group. This was in contrast to the DAG treatment group, which showed a similar level of aortic collagen content to that seen in control apoE^{-/-} mice. This suggests that AG may be providing its beneficial effects on aortic lesions by preventing both the deposition of vascular collagen and, subsequently, AGE cross-link formation. This is also supported by the results of assessment of individual collagen subtypes. It is interesting that neither drug removed collagen localized at the luminal surface of the plaques. It is tempting to speculate that this may be beneficial to plaque stability in light of recent evidence suggesting that collagen ratios (42) and fibromuscular cap composition (43) are important contributors to plaque stability. sRAGE admin-

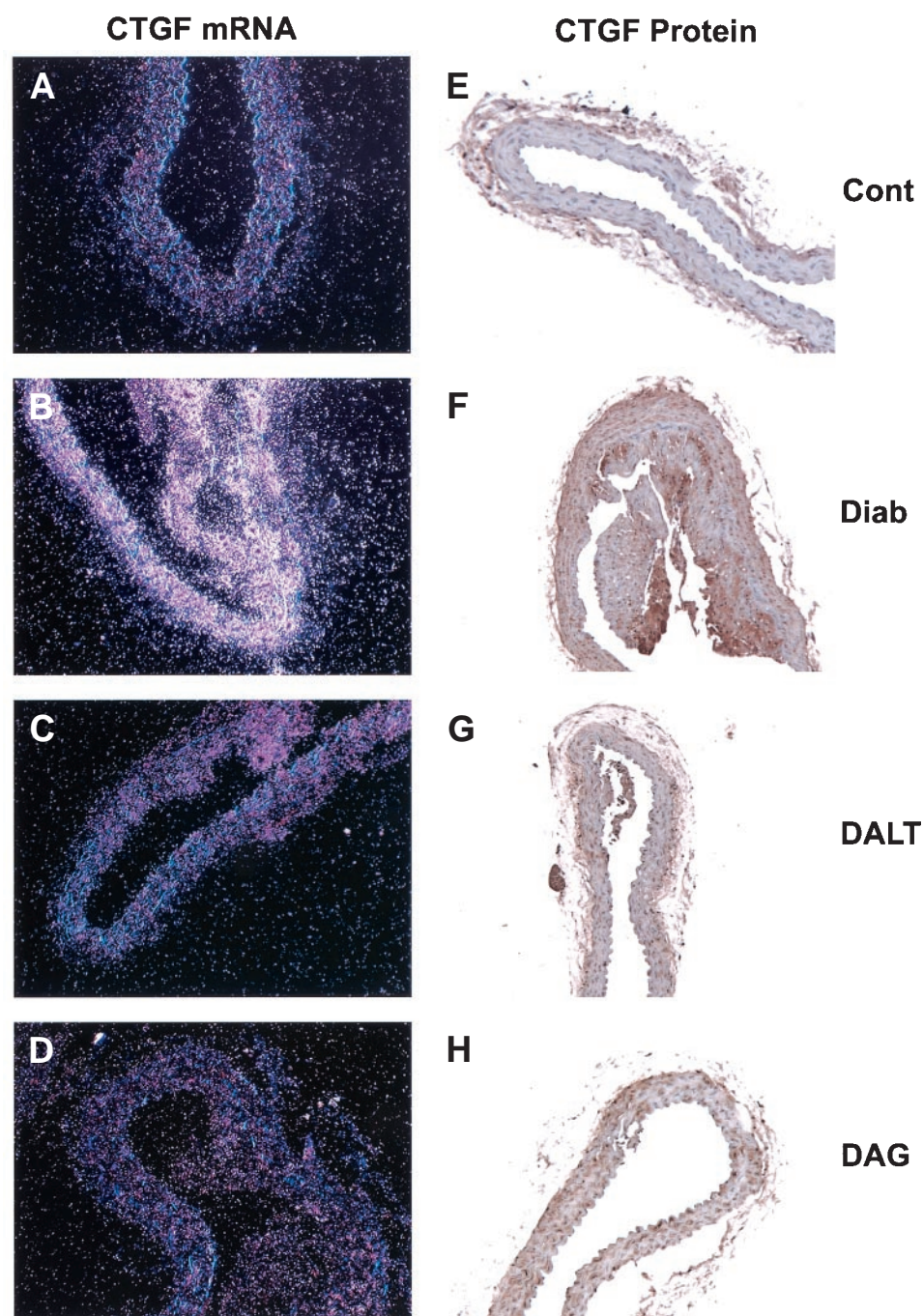


FIG. 6. Representative aortic dark field in situ hybridization for CTGF. For control (A), diabetes (B), DALT (C), and DAG (D); and aortic CTGF protein visualized by immunohistochemistry in control (Cont; E), diabetes (Diab; F), DALT (G), and DAG (H). Magnification $\times 200$.

istration in this model has also been reported to be associated with less collagen accumulation (37).

The present study demonstrated a blood pressure-lowering effect of ALT-711. This is consistent with preliminary clinical data from the as-yet-unpublished SILVER/SAPPHIRE trials in isolated systolic hypertension in which ALT-711 has conferred positive effects on blood pressure, albeit it in a nondiabetic context (www.alteon.com). One cannot exclude a role for blood pressure in the acceleration of atherosclerosis because our group has previously shown beneficial effects in the diabetic apoE $^{-/-}$ model with the ACE inhibitor perindopril (16). Although this effect of the ACE inhibitor has, in general, been interpreted as suggesting a role for blockade of the RAS as the mediator of reduced aortic

plaque formation, a direct effect of ALT-711 on blood pressure cannot be excluded. Nevertheless, AG also attenuated atherosclerosis without affecting systemic blood pressure. Furthermore, other studies have shown that hypertension does not account for accelerated atherosclerosis in apoE $^{-/-}$ mice in association with vascular dysfunction (44). Indeed, our own studies in diabetic apoE $^{-/-}$ mice that were treated with the calcium channel blocker amlodipine have identified no improvement in aortic lesions despite normalization of blood pressure (45). In support of this observation, studies by Miyata et al. (33) have also previously demonstrated that nifedipine, another dihydropyridine calcium channel blocker, has no effect on AGE formation.

The accumulation of collagen I was identified throughout the vessel wall and on the luminal side of plaques from diabetic apoE^{-/-} mice. It is interesting that although the increase in vascular collagen I accumulation was partially abrogated by treatment with ALT-711, the plaque content of collagen I remained unchanged. By contrast, AG attenuated the increases in both the aortic wall and the plaque for collagen I. Furthermore, diabetes-induced increases in collagen III and IV, although attenuated in the vascular wall, were not reduced within plaques from treated animals. Because the presence of collagen within the plaques may provide plaque stability (42), the region-specific depletion of collagen I within the vessel wall but not within the plaque itself by ALT-711, in contrast to depletion at both sites by AG, may ultimately represent a potential therapeutic advantage of ALT-711 over agents such as AG. However, interpretation of these findings remains speculative because this difference between agents was not noted for all collagen subtypes. In addition, AGEs have been shown to induce the proliferation (46) and activation of vascular smooth muscle cells (47) to produce fibrillar collagen. This is supported in the present study by the amelioration of diabetes-induced α -SMA expression by each of the AGE reducing treatments.

The profibrotic cytokine CTGF was increased in the atherosclerotic vessels and may be contributing to the deposition of matrix proteins in the vasculature of these diabetic apoE^{-/-} mice. Recent studies by our own group have demonstrated increases in aortic CTGF in this experimental model of atherosclerosis and diabetes (16). Furthermore, we have previously shown that AG (26) and ALT-711 (13) have the ability to reduce the expression of CTGF in diabetic nephropathy. Therefore, the reduction in CTGF expression in response to the inhibitors of AGE accumulation as demonstrated in the present study was not surprising. These findings provide further evidence for the view that this growth factor plays a pivotal role in mediating collagen deposition in various contexts, including diabetes.

RAGE has been identified as a modulator of inflammation (48) and as a major contributor to atherogenesis (10); therefore, not surprisingly, diabetes increased the expression of RAGE in the vascular wall in apoE^{-/-} mice. The reduction in aortic RAGE expression that was seen after ALT-711 or AG treatment has also been reported after administration of sRAGE in this same model (10). In all of these studies, the various treatments were associated with a reduction in atherosclerosis. It remains to be determined whether the reduction in RAGE is just a manifestation of less vascular disease or represents a central mechanism for mediating the antiatherosclerotic effects of different treatments that modulate the advanced glycation pathway. The benefits of sRAGE in this model have been interpreted as indicative of the direct participation of RAGE in the development of diabetes-associated atherosclerosis. However, an alternative interpretation of the results is that sRAGE is acting primarily as a ligand to bind AGEs, thereby promoting their removal and preventing their interaction with other receptors to promote vascular injury. RAGE also acts as a receptor to other inflammatory molecules such as S-100, which may also promote atherosclerosis (48). However, the findings of the present study

emphasize the benefits of blocking AGE accumulation per se and are consistent with a mechanism whereby sRAGE confers antiatherosclerotic effects via suppression of AGE-mediated pathways. The recent advent of a viable RAGE knockout mouse will greatly facilitate further elucidation of the specific role of RAGE in this field (49).

The present study has identified that AGEs play an important role in diabetes-associated atherosclerosis, because ALT-711 and AG both were effective in reducing both atherosclerotic plaque formation and complexity. These findings extend our understanding of the central role of AGEs in atherosclerosis, particularly in the diabetic context, and provide clinical investigators with an increasing number of therapeutic options to explore as part of the approach to reduce the burden of cardiovascular disease in diabetes.

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REFERENCES

- King GL, Wakasaki H: Theoretical mechanisms by which hyperglycemia and insulin resistance could cause cardiovascular diseases in diabetes. *Diabetes Care* 22 (Suppl 3):C31-C37, 1999
- Uusitupa MI, Niskanen LK, Siitonen O, Voutilainen E, Pyorala K: 5-Year incidence of atherosclerotic vascular disease in relation to general risk factors, insulin level, and abnormalities in lipoprotein composition in non-insulin-dependent diabetic and nondiabetic subjects. *Circulation* 82: 27-36, 1990
- Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232:1629-1632, 1986
- Vlassara H, Fuh H, Donnelly T, Cybulsky M: Advanced glycation endproducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. *Mol Med* 1:447-456, 1995
- Menzel EJ, Neumuller J, Sengoele G, Reihnsner R: Effects of aminoguanidine on adhesion molecule expression of human endothelial cells. *Pharmacology* 55:126-135, 1997
- Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D: Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice: a potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 96:1395-1403, 1995
- Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW: Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions: mechanism of action of pyridoxamine. *J Biol Chem* 275:21177-21184, 2000
- Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR, Baynes JW: Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61:939-950, 2002
- Alderson NL, Chachich ME, Youssef NN, Beattie RJ, Nachtigal M, Thorpe

- SR, Baynes JW: The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats. *Kidney Int* 63:2123–2133, 2003
10. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM: Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 4:1025–1031, 1998
 11. Schmidt AM, Yan SD, Yan SF, Stern DM: The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* 108:949–955, 2001
 12. Vasan S, Foiles P, Founds H: Therapeutic potential of breakers of advanced glycation end product-protein crosslinks. *Arch Biochem Biophys* 419:89–96, 2003
 13. Forbes JM, Thallas V, Thomas MC, Founds HW, Burns WC, Jerums G, Cooper ME: The breakdown of pre-existing advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. *FASEB J* 17:1762–1764, 2003
 14. Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC, Tikellis C, Ritchie RH, Twigg SM, Cooper ME, Burrell LM: A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res* 92:785–792, 2003
 15. Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroot RC, Lakatta EG: Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation* 104:1464–1470, 2001
 16. Candido R, Jandeleit-Dahm KA, Cao Z, Nesteroff SP, Burns WC, Twigg SM, Dille RJ, Cooper ME, Allen TJ: Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. *Circulation* 106:246–253, 2002
 17. Cefalu WT, Wang ZQ, Bell-Farrow A, Kiger FD, Izlar C: Glycohemoglobin measured by automated affinity HPLC correlates with both short-term and long-term antecedent glycemia. *Clin Chem* 40:1317–1321, 1994
 18. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499–502, 1972
 19. Krege JH, Hodgin JB, Hagaman JR, Smithies O: A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension* 25:1111–1115, 1995
 20. Monnier V, Cerami A: Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science* 211:491–494, 1981
 21. Nakamura K, Nakazawa Y, Ienaga K: Acid-stable fluorescent advanced glycation end products: vespersylines A, B, and C are formed as crosslinked products in the Maillard reaction between lysine or proteins with glucose. *Biochem Biophys Res Commun* 232:227–230, 1997
 22. Wrobel K, Garay-Sevilla ME, Nava LE, Malacara JM: Novel analytical approach to monitoring advanced glycosylation end products in human serum with on-line spectrophotometric and spectrofluorometric detection in a flow system. *Clin Chem* 43:1563–1569, 1997
 23. Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 91:2463–2469, 1993
 24. Stegemann H, Stalder K: Determination of hydroxyproline. *Clin Chim Acta* 18:267–273, 1967
 25. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29:577–580, 1981
 26. Twigg SM, Cao Z, McLennan SV, Burns WC, Brammar G, Forbes JM, Cooper ME: Renal connective tissue growth factor induction in experimental diabetes is prevented by aminoguanidine. *Endocrinology* 143:4907–4915, 2002
 27. Makita Z, Vlassara H, Cerami A, Bucala R: Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 267:5133–5138, 1992
 28. Forbes JM, Hewitson TD, Becker GJ, Jones CL: Ischemic acute renal failure: long-term histology of cell and matrix changes in the rat. *Kidney Int* 57:2375–2385, 2000
 29. Horiuchi S, Sano H, Higashi T, Ikeda K, Jinnouchi Y, Nagai R, Takahashi K: Extra- and intracellular localization of advanced glycation end-products in human atherosclerotic lesions. *Nephrol Dial Transplant* 11 (Suppl 5):81–86, 1996
 30. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA_{1c} as markers of diabetic complications: DCCT Skin Collagen Ancillary Study Group: Diabetes Control and Complications Trial. *Diabetes* 48:870–880, 1999
 31. Nathan DM, Lachin J, Cleary P, Orchard T, Brillion DJ, Backlund JY, O'Leary DH, Genuth S: Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. *N Engl J Med* 348:2294–2303, 2003
 32. Degenhardt TP, Thorpe SR, Baynes JW: Chemical modification of proteins by methylglyoxal. *Cell Mol Biol (Noisy-le-grand)* 44:1139–1145, 1998
 33. Miyata T, van Ypersele de Strihou C, Ueda Y, Ichimori K, Inagi R, Onogi H, Ishikawa N, Nangaku M, Kurokawa K: Angiotensin II receptor antagonists and angiotensin-converting enzyme inhibitors lower in vitro the formation of advanced glycation end products: biochemical mechanisms. *J Am Soc Nephrol* 13:2478–2487, 2002
 34. Forbes JM, Cooper ME, Thallas V, Burns WC, Thomas MC, Brammar GC, Lee F, Grant SL, Burrell LA, Jerums G, Osicka TM: Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy. *Diabetes* 51:3274–3282, 2002
 35. Corbett JA, Tilton RG, Chang K, Hasan KS, Ido Y, Wang JL, Sweetland MA, Lancaster JR Jr, Williamson JR, McDaniel ML: Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* 41:552–556, 1992
 36. Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL: Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice. *Circulation* 103:3099–3104, 2001
 37. Bucciarelli LG, Wendt T, Qu W, Lu Y, Lalla E, Rong LL, Goova MT, Moser B, Kislinger T, Lee DC, Kashyap Y, Stern DM, Schmidt AM: RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation* 106:2827–2835, 2002
 38. Vasan S, Zhang X, Kapurniotu A, Bernhagen J, Teichberg S, Basgen J, Wagle D, Shih D, Terlecky I, Bucala R, Cerami A, Egan J, Ulrich P: An agent cleaving glucose-derived protein crosslinks in vitro and in vivo. *Nature* 382:275–278, 1996
 39. Wolfenbuttel BH, Boulanger CM, Crijns FR, Huijberts MS, Poitevin P, Swennen GN, Vasan S, Egan JJ, Ulrich P, Cerami A, Levy BI: Breakers of advanced glycation end products restore large artery properties in experimental diabetes. *Proc Natl Acad Sci U S A* 95:4630–4634, 1998
 40. Mentink CJ, Hendriks M, Levels AA, Wolfenbuttel BH: Glucose-mediated cross-linking of collagen in rat tendon and skin. *Clin Chim Acta* 321:69–76, 2002
 41. Yang S, Litchfield JE, Baynes JW: AGE-breakers cleave model compounds, but do not break Maillard crosslinks in skin and tail collagen from diabetic rats. *Arch Biochem Biophys* 412:42–46, 2003
 42. Neumeister V, Scheibe M, Lattke P, Jaross W: Determination of the cholesterol-collagen ratio of arterial atherosclerotic plaques using near infrared spectroscopy as a possible measure of plaque stability. *Atherosclerosis* 165:251–257, 2002
 43. Shiomi M, Ito T, Hirouchi Y, Enomoto M: Fibromuscular cap composition is important for the stability of established atherosclerotic plaques in mature WHHL rabbits treated with statins. *Atherosclerosis* 157:75–84, 2001
 44. Chen J, Kuhlencordt PJ, Astern J, Gyurko R, Huang PL: Hypertension does not account for the accelerated atherosclerosis and development of aneurysms in male apolipoprotein e/endothelial nitric oxide synthase double knockout mice. *Circulation* 104:2391–2394, 2001
 45. Candido R, Allen TJ, Lassila M, Cao Z, Thallas V, Cooper ME, Jandeleit-Dahm K: Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis. *Circulation* 109:1536–1542, 2003
 46. Iino K, Yoshinari M, Yamamoto M, Kaku K, Doi Y, Ichikawa K, Iwase M, Fujishima M: Effect of glycated collagen on proliferation of human smooth muscle cells in vitro. *Diabetologia* 39:800–806, 1996
 47. Mizutani K, Ikeda K, Yamori Y: Resveratrol inhibits AGEs-induced proliferation and collagen synthesis activity in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. *Biochem Biophys Res Commun* 274:61–67, 2000
 48. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, Neurath MF, Slattery T, Beach D, McClary J, Nagashima M, Morser J, Stern D, Schmidt AM: RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 97:889–901, 1999
 49. Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, Bucciarelli LG, Rong LL, Moser B, Markowitz GS, Stein G, Bierhaus A, Liliensiek B, Arnold B, Nawroth PP, Stern DM, D'Agati VD, Schmidt AM: RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. *Am J Pathol* 162:1123–1137, 2003