J Am Soc Nephrol 15: 2125–2138, 2004

Accelerated Nephropathy in Diabetic Apolipoprotein E-Knockout Mouse: Role of Advanced Glycation End Products

MARKUS LASSILA,* KWEE K. SEAH,* TERRI J. ALLEN,* VICKI THALLAS,* MERLIN C. THOMAS,* RICCARDO CANDIDO,* WENDY C. BURNS,* JOSEPHINE M. FORBES,* ANNA C. CALKIN,† MARK E. COOPER,* and KARIN A.M. JANDELEIT-DAHM*

**Danielle Alberti Memorial Centre for Diabetes Complications, Vascular Division, Wynn Domain, Baker Heart Research Institute, Melbourne, Victoria, Australia; and †Department of Medicine, Central and Eastern Clinical School, Monash University, Alfred Hospital, Melbourne, Australia*

Abstract. Hyperlipidemia not only may be relevant to cardiovascular disease in diabetes but may also play a role in the development and progression of diabetic nephropathy. Furthermore, there is increasing evidence that advanced glycation end products (AGE) play an important role in diabetic renal disease. The objectives of this study were first to characterize renal injury in diabetic apolipoprotein E knockout (apo E-KO) mice and second to explore the role of AGE in the development and progression of renal disease in this model. Diabetes was induced by injection of streptozotocin in 6-wk-old apo E-KO mice. Diabetic animals received no treatment or treatment with the inhibitor of AGE formation aminoguanidine (1 g/kg per d) or the cross-link breaker [4,5-dimethyl-3-(2-oxo2-phenylethyl)-thiazolium chloride] ALT-711, which cleaves preformed AGE (20 mg/kg per d) for 20 wk. Nondiabetic apo E-KO mice as well as nondiabetic and diabetic C57BL/6 mice served as controls. Compared with nondiabetic apo E-KO mice, induction of diabetes in apo E-KO mice resulted in accelerated renal injury characterized by albuminuria and glomerular and tubulointerstitial injury. These abnormalities were associated with increased expression of collagen type I and type IV and transforming growth factor-β1 (TGF-β1), increased α-smooth muscle actin immunostaining and macrophage infiltration, and increased serum and renal AGE. The two treatments, which attenuated renal AGE accumulation in a disparate manner, were associated with less albuminuria, structural injury, macrophage infiltration, TGF-β1, and collagen expression. The accelerated renal injury that was observed in diabetic apo E-KO mice was attenuated by approaches that inhibit renal AGE accumulation.

Dyslipidemia contributes to the increased risk of death and morbidity in patients with diabetes by accelerating macrovascular disease (1). Furthermore, clinical observations have suggested that hyperlipidemia is a contributory factor to the progression of diabetic renal disease (2,3). Experimental studies with various animal models of hyperlipidemia, including apolipoprotein E knockout (apo E-KO) mice (4) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats (5), have shown that hyperlipidemia *per se* is associated with development of early renal lesions. The importance of hyperlipidemia in the progression of renal disease is supported by the findings that lipid reduction may confer renal protection (6). However, this issue remains controversial because some lipid-lowering drugs may have renoprotective effects, independent of their lipid-lowering effects (7).

Diabetes is characterized by chronic hyperglycemia, which accelerates the reaction between glucose and proteins, lipids, and nucleic acids and leads to the formation of advanced glycation end products (AGE). These chemically heterogeneous compounds have a wide range of chemical, cellular, and tissue effects implicated in the development and progression of diabetic nephropathy (8–10). Renal AGE accumulation can be blocked by inhibiting AGE formation with agents such as aminoguanidine or by cleavage of preformed AGE with a cross-link breaker such as ALT-711 [4,5-dimethyl-3-(2-oxo2-phenylethyl)-thiazolium chloride]. Such interventions that reduce renal AGE accumulation have been shown to confer renoprotection in the context of diabetes (8,11).

AGE mediate their effects through a number of different pathways (8). The formation of inter- and intramolecular cross-links after AGE modification leads to functional and structural alterations of renal proteins in the kidney. AGE also directly interact with a variety of receptors in the kidneys, which may lead to activation of several inflammatory pathways and growth factors such as TGF-β1 (12,13).

An earlier study using diabetic apo E-KO mice reported that

Received November 9, 2003. Accepted May 3, 2004.

Correspondence to Dr. Karin A.M. Jandeleit-Dahm, Baker Heart Research Institute, PO Box 6492, Commercial Road, Melbourne 8008, VIC 3004, Australia. Phone: +61-3-9276-2983; Fax: +61-3-8532-1288; E-mail: karin.jandeleit-dahm@baker.edu.au

1046-6673/1508-2125
Journal of the American Society of Nephrology
Copyright © 2004 by the American Society of Nephrology

DOI: 10.1097/01.ASN.0000133025.23732.46

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

relatively short-term diabetes (6 wk) resulted in increased renal expression of the receptor for AGE (RAGE) and infiltration of inflammatory cells (14). Soluble RAGE administration reduced infiltration of inflammatory cell into the kidneys from diabetic animals, although detailed functional and structural effects were not described (14).

The aim of the present study was to characterize renal injury in a model combining long-term diabetes and hyperlipidemia, the diabetic apo E-KO mouse. It was hypothesized that this model would demonstrate accelerated renal injury and that AGE would play a major role in promoting renal injury in this model. The present study used two disparate approaches to inhibit renal AGE accumulation in this model: an inhibitor of AGE formation, aminoguanidine, and the putative cross-link breaker ALT-711, which cleaves preformed AGE (15).

Materials and Methods

Six-week-old homozygous apo E-KO male mice (back-crossed 20 times from the C57BL/6 strain; Animal Resource Centre, Canning Vale, W.A., Australia) and normal C57BL/6 mice were housed at the Biologic Research Laboratory at the Austin and Repatriation Medical Centre. These animals were studied according to the principles devised by the Animal Welfare Committee of the Austin and Repatriation Medical Centre. Fifty-three apo E-KO and 20 C57BL/6 mice were rendered diabetic by five daily intraperitoneal injections of streptozotocin (Boehringer-Mannheim, Mannheim, Germany) at a dose of 55 mg/kg in citrate buffer (16). Only animals with blood glucose levels >15 mmol/L 2 d after the induction of diabetes were included in the study. Control mice ($n = 20$ in each strain) received citrate buffer alone. The animals had unrestricted access to water and were maintained on a 12-h light-dark cycle in a non–pathogen-free environment on standard mouse chow (Barastoc, Pakenham, VIC, Australia). After the induction of diabetes, the animals were further randomized to receive either no treatment ($n = 20$) or treatment with aminoguanidine (Fluka Pharmaceuticals, Buchs SG, Switzerland) at a dose of 1 g/kg body wt per d in drinking water ($n = 20$) or ALT-711 (Alteon, Ramsey, NJ) at a dose of 20 mg/kg body wt per d by gavage ($n = 13$). Systolic BP was assessed by a noninvasive tail cuff system in conscious mice at the end of the study (17). Animals were habituated to the device before measuring the BP to ensure accurate measurements.

After 20 wk, the animals were anesthetized by an intraperitoneal injection of pentobarbitone sodium (55 mg/kg body wt; Nembutal;, Boehringer Ingelheim, Artarmon, NSW, Australia). Blood was collected from the left ventricle and centrifuged, and plasma and red blood cells were stored at −20°C and at 4°C, respectively, for subsequent analyses. Glycated hemoglobin (HbA_{1c}) was determined in lysates of red blood cells by HPLC (Bio-Rad, Richmond, CA). Total cholesterol and triglyceride concentrations were measured by autoanalyzer (Hitachi 917, Tokyo, Japan). The mouse kidneys were rapidly dissected and snap-frozen in liquid nitrogen and stored at −80°C or stored in buffered formalin (10%) for subsequent immunohistochemical studies.

Assessment of Renal Function

At week 20, the animals were housed in metabolic cages for 24 h for collection of urine samples for the later measurements of albumin concentration by RIA. This assay is a modification of a previously established method for measuring rat albumin in our laboratory (18). In brief, a rabbit anti-mouse albumin antibody (Cappel, Aurora, OH) was used followed by a second antibody, a sheep anti-rabbit antibody (ProSearch, Melbourne, Australia). Plasma urea and creatinine concentrations at the end of the study were measured by autoanalyzer (Beckman Instruments, Fullerton, CA).

Evaluation of Morphologic Changes

Two-micrometer-thick cross-sections of the kidneys were prepared and stained with periodic acid-Schiff to evaluate renal pathology. Assessment of the amount of glomerular and tubulointerstitial injury was performed in a blinded manner using a semiquantitative method (19). Glomerular injury included mesangial matrix expansion and/or hyalinosis with focal adhesions, capillary dilation, and true glomerular tuft occlusion and sclerosis. The assessment of tubulointerstitial injury included tubular dilation, interstitial fibrosis, and tubular cell atrophy. In brief, 40 glomeruli in each kidney were graded according to the severity and amount of the glomerular injury: grade 0, intact glomerulus; grade 1, glomerular injury involving <25% of the glomerulus; grade 2, glomerular injury affecting 25 to 50% of the glomerulus; grade 3, glomerular injury affecting ~50 to 75% of the glomerulus; grade 4, severe damage or sclerosis involving >75% of the glomerulus. For tubulointerstitial injury, 40 tubulointerstitial fields were graded according to the severity and amount of the tubular and interstitial damage: grade 0, no tubular injury, no interstitial fibrosis; grade 1, <25% of the field involved; grade 2, 25 to 50% of the field involved; grade 3, 50 to 75% of the field involved; grade 4, >75% of the tubules and interstitium per field involved. The indices for glomerular and tubulointerstitial injury were calculated using the following formula:

$$GSI/TI = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)/(n_0 + n_1 + n_2 + n_3 + n_4)$$

where n_x = number of glomeruli/tubulointerstitial fields in each grade of glomerular/tubulointerstitial injury. These indices of glomerular/tubulointerstitial injury were calculated by averaging the grades assigned to all glomeruli/tubulointerstitial fields (20). For assessing renal lipid accumulation, frozen renal sections (6 μm) were stained for lipids with Sudan IV-Herxheimer's solution (Sigma Chemical Co., St. Louis, MO) (16).

Immunohistochemistry

Frozen renal sections (6 μm) were used to immunostain for macrophages with a rat anti-mouse F4/80 antibody (Serotec, Oxford, UK; diluted 1:50) (21). In brief, frozen sections were fixed with cold acetone and blocked with normal rabbit serum (1/5 in Tris-buffered saline [TBS]). The sections were incubated with the primary antibody overnight at 4°C. Endogenous nonspecific binding for biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), and endogenous peroxidase was inactivated using 0.6% H_2O_2 in TBS. Biotinylated rabbit anti-rat Ig (Vector Laboratories), diluted 1:200 in TBS, was used as the secondary antibody for 60 min, followed by Vectastain ABC reagent (Vector Laboratories) for 30 min. Peroxidase activity was identified by reaction with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) as the chromogen. The slides were then counterstained with hematoxylin, dehydrated, and mounted.

Two-micrometer paraffin sections of kidneys were used to stain for α-smooth muscle actin [α-SMA], collagen type I and IV, TGF-β1, AGE, and RAGE. The primary antibodies that were used included a monoclonal mouse anti-human α-SMA antibody (Dako A/S, Copenhagen, Denmark; diluted 1:50), a polyclonal goat anti-collagen type IV antibody (diluted 1:1600; Southern Biotechnology, Birmingham, AL), a polyclonal goat anti-collagen type I antibody (diluted 1:200; Southern Biotechnology), a polyclonal rabbit anti–TGF-β1 antibody

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

(diluted 1:400; Santa Cruz Biotechnology, Santa Cruz, CA), a monoclonal mouse anti-mouse AGE antibody (AGE 4G9, which recognizes the AGE carboxymethyllysine [CML]; diluted 1:100; a gift from Dr. H. Founds, Alteon, Ramsey, NJ), and a polyclonal goat anti-RAGE antibody (diluted 1:250; Chemicon, Temecula, CA). In brief, sections for α-SMA and AGE were dewaxed, hydrated, and quenched with 3% H_2O_2 in PBS (pH 7.6) to inhibit endogenous peroxidase activity. This was followed by incubation in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, PA) for 30 min at room temperature. The sections were then incubated with anti–α-SMA, anti-AGE, or goat anti-RAGE antibodies overnight at 4°C. Sections for TGF-β1 were first heated, dewaxed, and then underwent pressure-cooker microwaving in 0.01 M citrate buffer at pH 6.0. This was followed by incubation with 3% H_2O_2 in PBS (pH 7.6) and Protein Blocking Agent and incubation with the primary antibody for TGF-β1 for 1 h at room temperature. For type I and type IV collagen, dewaxed and hydrated sections were quenched with 3% H_2O_2 in PBS. In addition, sections were digested with 0.4% pepsin (Sigma Chemical Co.) in 0.01 M HCl at 37°C. This was followed by incubation with 1% normal horse serum diluted in TBS to block nonspecific binding. Subsequently, sections were incubated with the primary antibody for either type I or type IV collagen overnight at 4°C in a humid atmosphere followed by avidin/biotin blocking. Thereafter, biotinylated anti-mouse Ig (Vector Laboratories, Burlingame, CA), diluted 1:250 for α-SMA and AGE, biotinylated anti-rabbit Ig (Vector Laboratories), diluted 1:500 for TGFβ1, or anti-goat, diluted 1:500 for collagen type I and IV and RAGE, was applied as the secondary antibody, followed by horseradish peroxidase–conjugated streptavidin (VECTASTAIN Elite ABC Staining Kit, Vector Laboratories). Peroxidase conjugates were subsequently visualized using 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 0.08% H_2O_2/PBS as the chromogen. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

All sections were examined under light microscopy (Olympus BX-50, Olympus Optical) and digitized with a high-resolution camera. For the quantification of proportional area of staining, ~20 views were randomly located in the renal cortex and corticomedullary junction of each slide (Optimas 6.2-Video Pro-32; Bedford Park, SA, Australia). For some parameters with obvious differences detected with respect to glomerular, tubular, and interstitial expression, separate analyses were performed for these individual renal compartments. All assessments were performed in a blinded manner.

AGE Peptides in Serum

Fluorescence AGE peptides in serum were assayed using HPLC with on-line spectrofluorometric detection (22,23). Spectrofluorometric detection of AGE peptides was measured at 440 nm with excitation at 247 nm. Samples were run in triplicate, and the area under the curve was used for signal measurements. The assay was calibrated against AGE peptide obtained from enzymatic hydrolysis of AGE-BSA (10 g/L). Total peptide content was estimated using an on-line spectrophotometric detector set to a wavelength of 280 nm.

Reverse Transcription–PCR

Six micrograms of total RNA extracted from a whole kidney using Trizol method were used to synthesize cDNA with Superscript First Strand synthesis system for reverse transcription–PCR (Life Technologies BRL, Grand Island, NY) (24). RAGE, AGE receptor-2 (AGE R-2), and AGE R-3 gene expression were analyzed by real-time quantitative reverse transcription–PCR using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer, Foster City, CA) (24). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems). For controlling for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700, Perkin-Elmer). Primers and Taqman probes for the target sequences and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700, Perkin-Elmer).

For amplification of the RAGE cDNA, the forward primer was 5'-GGGAAGGAGGTCAAGTCCAACTA-3' and the reverse primer was 5'-TGAGTTCAGAGGCAGGATCCA-3'. The probe specific to RAGE was 5'-TCTACCAGATTCCTGGGAAGCCAGAAATTG-3'. For the AGE R-2 cDNA, the forward primer was 5'-ACGAGCTCACCACCAATGAGT-3' and the reverse primer was 5'-TTTGGGTTTCTGGGAGACCA-3'. The probe specific to AGE R-2 was 5'-CGTCTACCGGCTTTGCCCCTTCA-3'. For the AGE R-3 cDNA, the forward primer was 5'-CAGACAGCTTTTCGCTTAACGA-3' and the reverse primer was 5'-CCATGCACCCGGATATCC-3'. The probe specific to AGE R-3 was FAM-5'-TTAGCTGGCTCTGGAAACCCAAACCCT-3'-TAMRA. Every amplification was performed with the following time course: 50°C, 2 min and 10 min at 95°C and 40 cycles of 94°C, 20 s, 60°C, 1 min. Each sample was tested in triplicate. Results are expressed relative to values in the control apo E-KO group, which were arbitrarily assigned a value of 1.

Statistical Analyses

Data were analyzed by ANOVA using Statview V (Brainpower, Calabasas, CA). Comparisons of group means were performed by Fisher least significant difference method. Data are shown as mean ± SEM, unless otherwise specified. $P < 0.05$ was viewed as statistically significant.

Results

Metabolic Parameters and Systolic BP

There was no difference in body weight gain over the 20-wk period between nondiabetic C57BL/6 and apo E-KO mice, but diabetic mice of both strains gained less weight than nondiabetic mice (Table 1). In both mouse strains, diabetes was associated with an increase in HbA_{1c} levels (Table 1). Plasma cholesterol levels were increased in nondiabetic apo E-KO mice when compared with the C57BL/6 mice, whereas triglyceride levels were similar in nondiabetic animals of both strains (Table 1). Diabetes was associated with a further increase in plasma cholesterol and triglyceride levels in diabetic apo E-KO mice but not in C57BL/6 mice. Systolic BP was modestly increased in apo E-KO mice when compared with C57BL/6 mice, but the induction of diabetes did not alter systolic BP significantly in either strain (Table 1).

Aminoguanidine or ALT-711 treatments had no significant effect on body weight, HbA_{1c}, triglyceride levels, or systolic BP when compared with untreated diabetic apo E-KO mice (Table 1). Treatment with ALT-711 but not aminoguanidine was associated with a modest decrease in plasma cholesterol, albeit the levels remained markedly elevated.

Renal Parameters

Nondiabetic apo E-KO mice had higher kidney/body weight ratios than C57BL/6 mice. Diabetes in both strains was asso-

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

Table 1. Biological parameters at the end of the 20-week study

Parameters	Control C57BL/6 (n = 20)	Diabetic C57BL/6 (n = 20)	Control apo E-KO (n = 20)	Diabetic apo E-KO (n = 20)	Diabetic apo E-KO + Aminoguanidine (n = 20)	Diabetic apo E-KO + ALT-711 (n = 13)
Body weight (g)	31 ± 1	23 ± 1[c]	30 ± 1	21 ± 0.3[c]	22 ± 1	21 ± 1
Kidney weight/body weight (g/kg)	6.42 ± 0.21	9.17 ± 0.16[c]	7.27 ± 0.50	8.78 ± 0.45[b]	8.16 ± 0.23	7.71 ± 0.35
HbA_{1c} (%)	3.3 ± 0.3	13.0 ± 1.0[c]	3.8 ± 0.1	13.0 ± 0.4[c]	12.1 ± 0.4	14.4 ± 1.1[h]
SBP (mmHg)	100 ± 3	98 ± 4	117 ± 2[e]	117 ± 5[e]	125 ± 3	123 ± 3
Plasma cholesterol (mmol/L)	1.8 ± 0.2	2.0 ± 0.2	14.3 ± 0.4[e]	35.6 ± 2.4[c,e]	31.6 ± 1.2	28.6 ± 2.2[f]
Plasma triglycerides (mmol/L)	0.76 ± 0.09	1.152 ± 0.20[b]	0.89 ± 0.07	1.86 ± 0.29[c,d]	2.00 ± 0.30	1.43 ± 0.20
Plasma creatinine (μmol/L)	16 ± 2	21 ± 3	13 ± 2	35 ± 3[c,d]	30 ± 2	37 ± 2
Plasma urea (mmol/L)	8.8 ± 1.5	13.8 ± 1.3[b]	8.7 ± 0.6	12.3 ± 1.1[b]	13.5 ± 1.1	13.5 ± 0.5

[a] Data are mean ± SEM. apo E-KO, apolipoprotein E knockout; SBP, systolic BP; HbA_{1c}, glycated hemoglobin.
[b] $P < 0.05$, [c] $P < 0.01$ *versus* appropriate nondiabetic control group.
[d] $P < 0.05$, [e] $P < 0.01$ *versus* appropriate C57BL/6 group.
[f] $P < 0.05$, [g] $P < 0.01$ *versus* diabetic apo E-KO mice.
[h] $P < 0.01$ *versus* diabetic apo E-KO mice treated with aminoguanidine.

ciated with an increase in the kidney/body weight ratio, but no difference in this parameter was detected between diabetic apo E-KO and diabetic C57BL/6 mice. Plasma urea and creatinine concentrations were similar in nondiabetic mice of either strain (Table 1). Diabetes was associated with increased serum urea concentrations in both strains, but serum creatinine concentrations were increased by diabetes in apo E-KO mice only. There was no difference in urinary albumin excretion rate between nondiabetic apo E-KO mice and C57BL/6 mice (Figure 1A). In both strains, induction of diabetes significantly increased urinary albumin excretion. The highest albumin excretion rate was seen in diabetic apo E-KO mice, which showed increased albuminuria when compared with both diabetic C57BL/6 mice and nondiabetic apo E-KO mice (Figure 1A).

Treatment with aminoguanidine or ALT-711 did not significantly affect kidney/body weight ratio, plasma urea, or creatinine concentrations when compared with untreated diabetic apo E-KO mice (Table 1). However, the increase in albuminuria was attenuated by treatment with either aminoguanidine or ALT-711 (Figure 1A).

In nondiabetic C57BL/6 mice, renal structure was preserved. In nondiabetic apo E-KO mice, there was a slight but significant increase in the glomerular injury index as well as in the tubulointerstitial injury index (Figure 1, B and C). Glomerular changes were characterized by deposits of lipids in the mesangium and in the capillaries, slight glomerular hyperplasia, deposition of extracellular matrix (ECM) in the mesangium associated with mesangial expansion, and mesangial hypercellularity. Tubulointerstitial changes included macrophage infiltration and a modest increase in interstitial space as a result of deposition of ECM. The induction of diabetes was associated with an increase in the degree and severity of the glomerular and tubular changes compared with nondiabetic apo E-KO mice. These changes included glomerular capillary dilation, increased capillary and mesangial lipid deposits, and increased mesangial expansion with increased infiltration by inflammatory cells and true glomerulosclerosis with glomerular tuft occlusion (Figure 1D). There was not only an increase in tubulointerstitial lesions but also an increase in their severity. These changes included increased infiltration of mononuclear cells, tubulointerstitial ECM deposition, and dilation of proximal and distal tubules and occasional tubular cell atrophy (Figure 1E). The indices for glomerular and tubulointerstitial injury were greater in diabetic apo E-KO mice when compared with diabetic C57BL/6 mice (Figure 1, B and C). Treatment with aminoguanidine or ALT-711 was associated with amelioration of these renal pathologic changes with reduced glomerular and tubulointerstitial injury indices in both treatment groups (Figure 1, B and C).

Glomerular Lipid Accumulation

In nondiabetic or diabetic C57BL/6 mice, no staining for Sudan IV was observed. In contrast, nondiabetic apo E-KO mouse kidneys showed clear lipid accumulation (0.74 ± 0.20%). In diabetic apo E-KO mice, there was a significant increase in glomerular lipid accumulation (1.72 ± 0.46%; $P < 0.05$ *versus* nondiabetic apo E-KO mice). Aminoguanidine

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

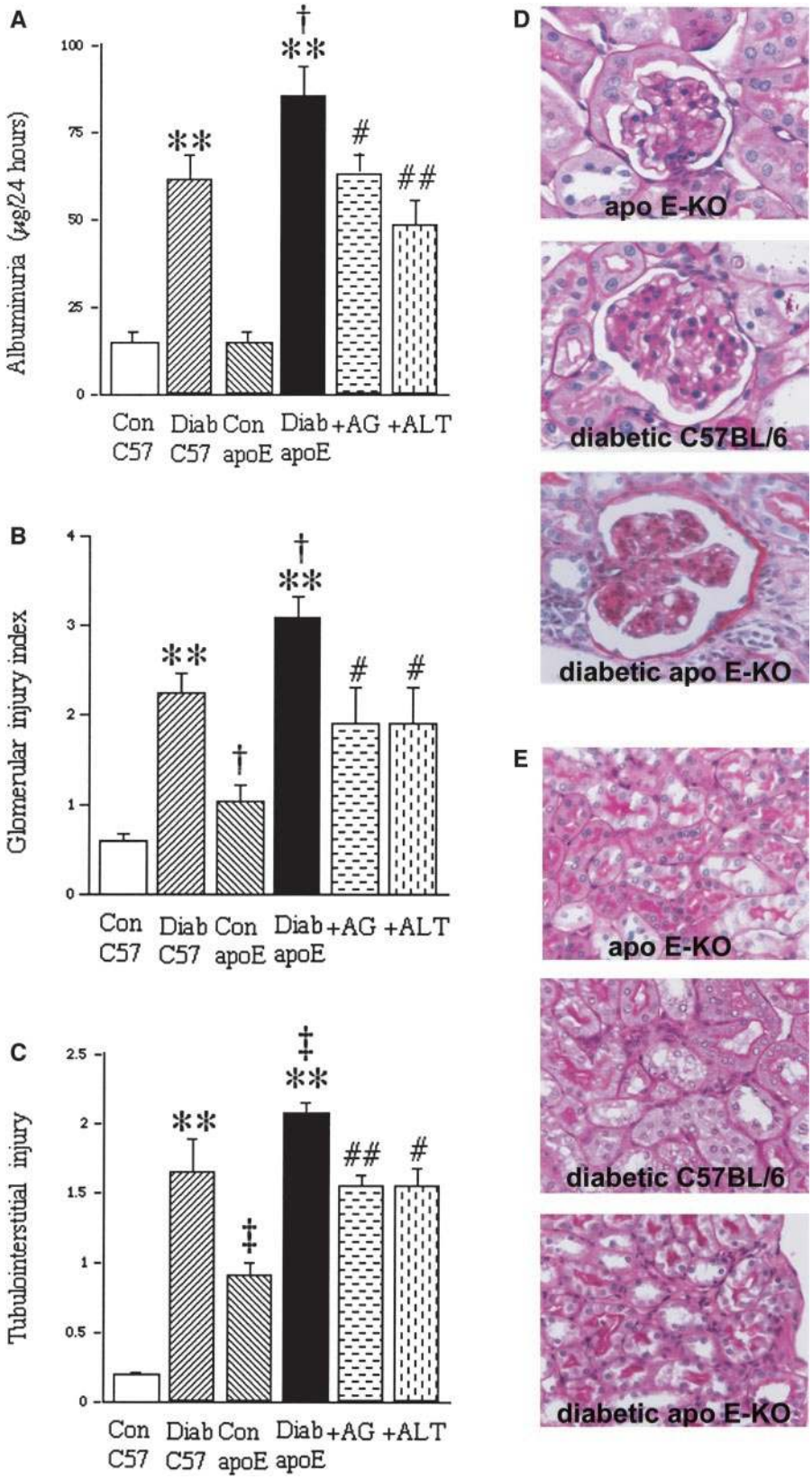

Figure 1. Albuminuria (A), glomerular injury index (B), and tubulointerstitial injury (C) in mice after the 20-wk treatment period. Representative pictures of glomerular (D) and tubulointerstitial (E) injury in nondiabetic apo E-KO, diabetic C57BL/6, and diabetic apo E-KO mice. Data are shown as mean ± SEM. $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *versus* appropriate C57BL/6 group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice. Magnifications: ×400 in D, ×200 in E.

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

(1.63 ± 0.40%) or ALT-711 (1.53 ± 0.46%) had no effect on glomerular lipid accumulation.

Macrophage Infiltration

In the nondiabetic animals, macrophage infiltration, as assessed by F4/80-positive cells, was observed in the interstitium with virtually no macrophages detected in glomeruli (Figure 2). Diabetes was associated with increased interstitial and glomerular infiltration of macrophages in both mouse strains (Figure 2). Treatment with aminoguanidine or ALT–711 was associated with a significant decrease in macrophage infiltration in both the glomerulus and the tubulointerstitium (Figure 2).

Expression of α-SMA–Positive Cells

In the nondiabetic mouse, renal immunostaining for α-SMA was essentially confined to renal arterioles and arteries, with only a little staining observed in the peritubular interstitium (Figure 3). Nondiabetic apo E-KO mice showed a slight increase in α-SMA expression in glomeruli and the tubulointerstitium when compared with C57BL/6 mice (Figure 3). In kidneys of diabetic mice, α-SMA expression was increased in glomeruli and in the tubulointerstitium when compared with nondiabetic mice. There was

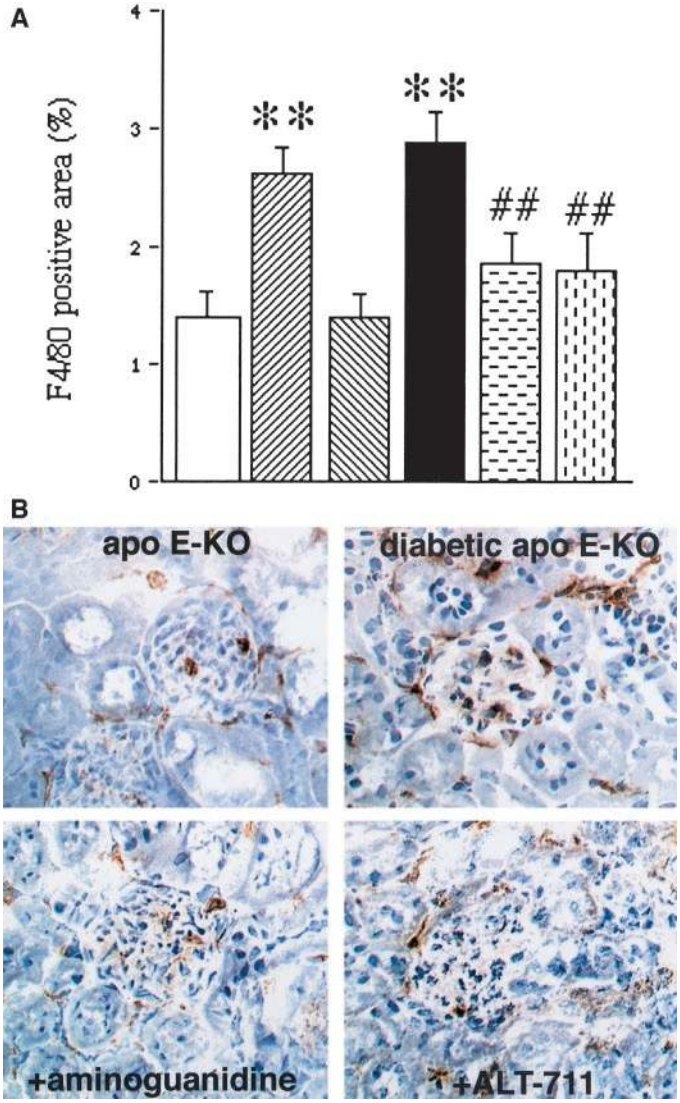

Figure 2. F4/80-positive macrophage staining in frozen section of the kidneys. (A) Quantification. (B) Representative pictures. Diabetic apo E-KO mice show increased macrophage infiltration in the glomerulus and the interstitium. Treatment with aminoguanidine or ALT-711 reduced macrophage infiltration. Data are shown as mean ± SEM. $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice. Magnification, ×200 in B.

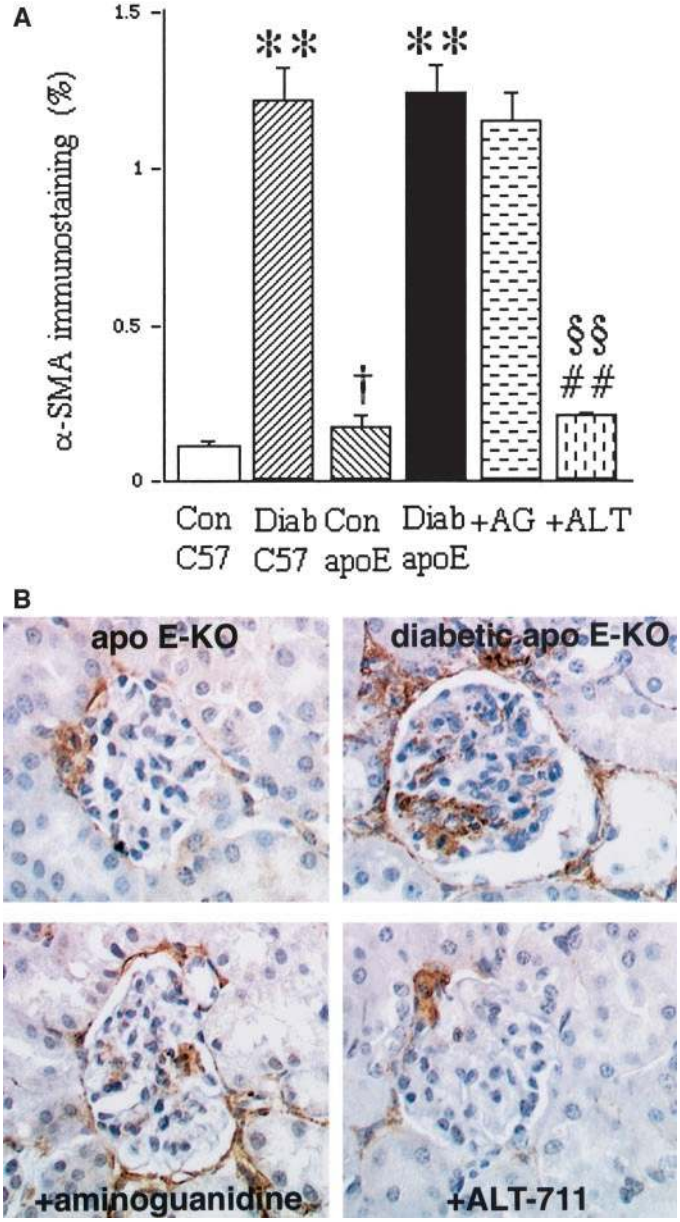

Figure 3. Quantification (A) and representative pictures (B) of immunohistochemistry for α-smooth muscle actin in the kidneys. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\dagger}P < 0.05$ *versus* appropriate C57BL/6 group; $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice; $^{\S\S}P < 0.01$ *versus* diabetic apo E-KO mice treated with aminoguanidine. Magnification, ×400 in B.

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

no significant difference between diabetic apo E-KO mice and diabetic C57BL/6 mice. In the diabetic kidneys, α-SMA–positive cells were detected in virtually all glomeruli, particularly in the mesangial cells as well as in the interstitium and occasionally in tubular cells. Treatment with ALT-711 prevented the increase in α-SMA–positive cell expression in diabetic apo E-KO mice (Figure 3). By contrast, aminoguanidine treatment had no effect on this parameter.

Type I and IV Collagen Expression

There was no difference in collagen type I expression between nondiabetic C57BL/6 and apo E-KO mice (Figure 4, A and B), whereas collagen type IV was increased in nondiabetic apo E-KO when compared with nondiabetic C57BL/6 mice (Figure 4, C and D). The induction of diabetes was associated with a similar increase in collagen type I and type IV protein expression in both mouse strains (Figure 4). The increase in collagen expression in diabetic kidneys was predominantly detected in the glomeruli as well as in the peritubular interstitium (Figure 4). Treatment with aminoguanidine or ALT-711 reduced accumulation of collagen type I and type IV, with ALT-711 treatment being associated with a more prominent reduction in collagen type I and IV protein expression than aminoguanidine treatment (Figure 4).

Fluorescence AGE in Serum

Fluorescence-labeled serum AGE levels were higher in nondiabetic apo E-KO than in nondiabetic C57BL/6 mice (Figure 5A).

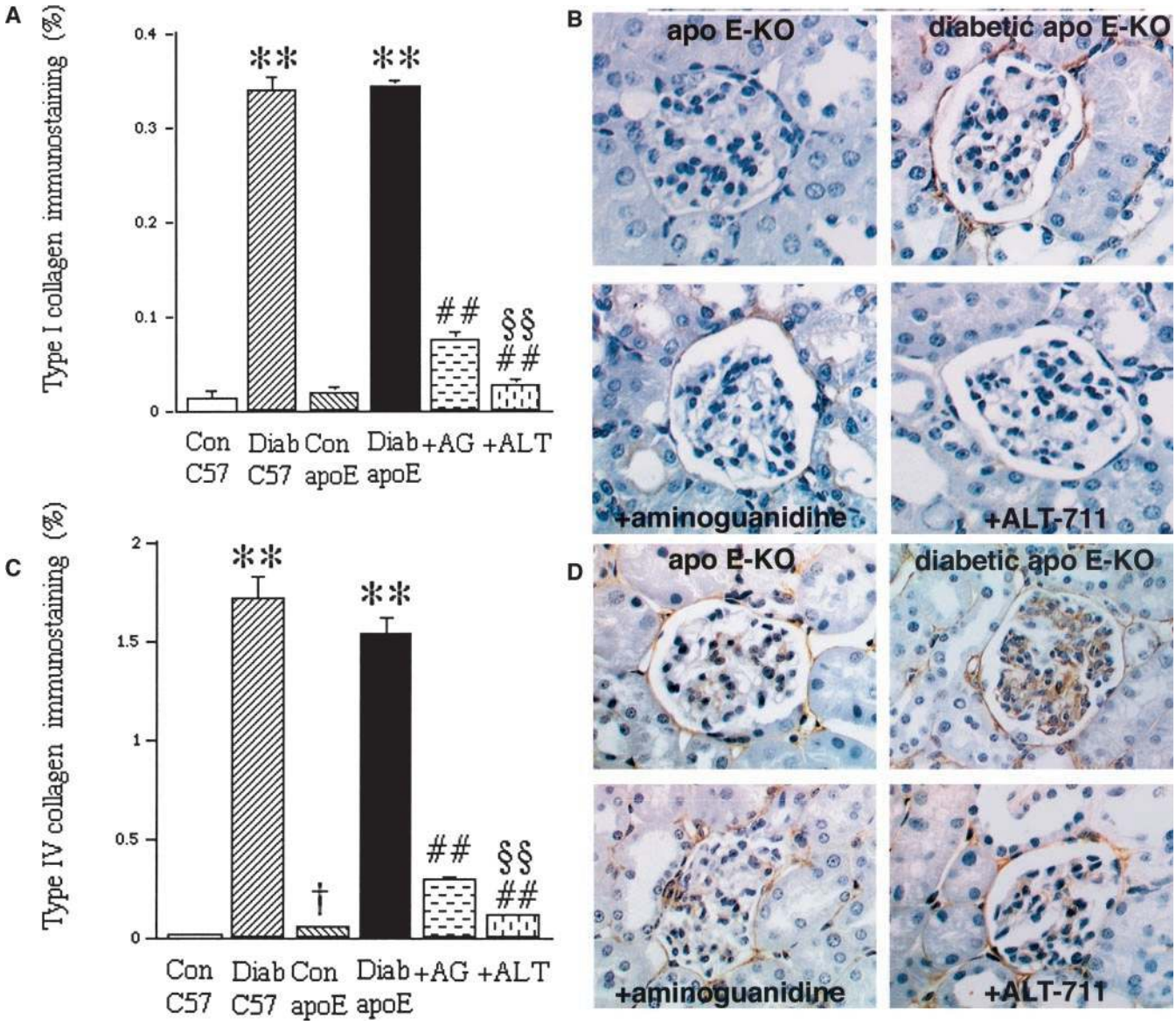

Figure 4. Immunohistochemistry for type I collagen (quantification [A] and representative pictures [B]) as well as type IV collagen (quantification [C] and representative pictures [D]) expression in the kidneys.. Data are shown as mean ± SEM. $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\dagger}P < 0.05$ *versus* appropriate C57BL/6 group; $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice; $^{\S\S}P < 0.01$ *versus* diabetic apo E-KO mice treated with aminoguanidine. Magnification, ×400 in B and D.

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

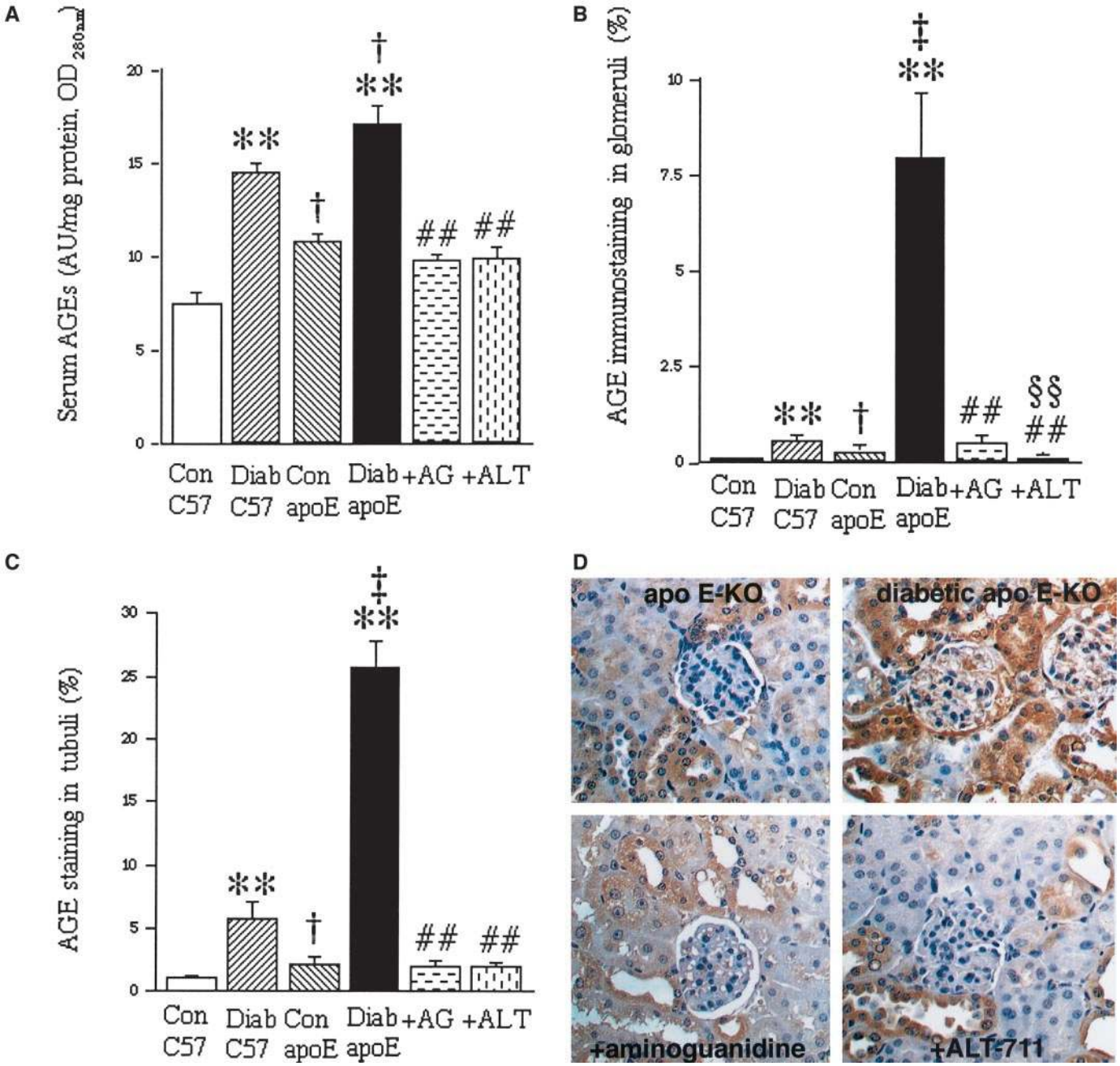

Figure 5. Quantification of fluorescence advanced glycation end products (AGE) in serum (A) and AGE immunostaining (carboxymethyllysine) in the kidneys (glomerulus quantification [B]; tubulus quantification [C]; representative pictures [D]). Data are shown as mean ± SEM. $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *versus* appropriate C57BL/6 group; $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice; §§$P < 0.01$ *versus* diabetic apo E-KO mice treated with aminoguanidine. Magnification, ×200 in D.

Diabetes was associated with increased serum AGE levels in both mouse strains, with the highest levels detected in the diabetic apo E-KO group. Aminoguanidine and ALT-711 treatment decreased serum AGE to levels similar to those observed in nondiabetic apo E-KO mice (Figure 5A).

AGE Immunostaining in the Kidney

AGE immunostaining (CML) was detected in the kidneys of C57BL/6 mice and was mainly observed in proximal and distal tubuli (Figure 5, B through D). In apo E-KO mice, there was in increase in AGE staining in tubuli, and some AGE staining was also detected in glomeruli (Figure 5, B through D). Induction of diabetes in both strains was associated with a marked increase in AGE immunostaining in both the glomerulus and the tubulointerstitium. Renal AGE immunostaining was significantly higher in diabetic apo E-KO mice than in diabetic C57BL/6 mice. Renal AGE expression was reduced to control apo E-KO levels by treatment with either aminoguanidine or ALT-711 (Figure 5, B and C).

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

Renal AGE Receptor Expression

Gene expression of the AGE receptors RAGE and AGE R-3 was increased in diabetic kidneys in both mouse strains to a similar degree (Table 2). No changes in renal AGE R-2 gene expression in diabetic kidneys were detected (Table 2). Immunohistochemical analysis showed that RAGE protein was increased in glomerular and tubular cells of nondiabetic apo E-KO mice. Induction of diabetes caused a further increase in RAGE immunostaining, but there was no difference between the diabetic groups of both strains (Table 2).

Treatment with aminoguanidine or ALT-711 was associated with decreased renal RAGE and AGE R-2 gene expression when compared with the untreated diabetic apo E-KO mice (Table 2). No significant effect on AGE R-3 gene expression was detected with either treatment (Table 2). Both treatments decreased RAGE protein expression when compared with untreated diabetic apo E-KO mice (Table 2).

Renal TGF-β1 Expression

In the kidneys of nondiabetic C57BL/6 mice, there was minimal staining for TGF-β1 (Figure 6). In nondiabetic apo E-KO mice, positive staining for TGF-β1 was seen in the glomeruli as well as in the tubulointerstitial areas (Figure 6). Diabetes was associated with a prominent increase in renal TGF-β1 immunostaining, which was seen in the glomerular epithelial and mesangial cells as well as in the tubulointerstitium. Tubulointerstitial TGF-β1 staining was significantly higher in diabetic apo E-KO mice than in diabetic C57BL/6 mice, whereas no difference in glomerular TGF-β1 staining was observed between these two diabetic groups (Figure 6). Treatment with ALT-711 or aminoguanidine reduced TGF-β1 expression in the glomeruli as well as in the tubulointerstitium (Figure 6) to levels similar to that observed in nondiabetic apo E-KO mice.

Discussion

The present study has demonstrated accelerated renal injury in a model of experimental diabetic nephropathy in the apo E-KO mouse. The renal injury was attenuated by inhibition of AGE accumulation by either an inhibitor of AGE formation or by a cross-link breaker, suggesting that AGE may play a pivotal role in diabetic renal injury in this model. Furthermore, attenuation of renal injury by AGE inhibitors was associated with reduced expression of a range of profibrotic and proinflammatory parameters.

AGE accumulation in both the serum and the kidney was increased in diabetic apo E-KO mice when compared with mice with diabetes or hyperlipidemia alone. In the serum, fluorescence AGE peptides, and in the kidney, increased deposition of the specific nonfluorescence AGE, CML, using an immunohistochemical approach were observed. CML has been reported to be the major AGE in mesangium, glomerular basement membrane, and tubular basement membrane (25) and has been shown to correlate with the severity of nephropathy in diabetic patients (25,26). CML is considered by some investigators to be a highly relevant and pathogenic advanced glycation/lipoxidation end product and is formed by both glucose-

Table 2. Renal expression of receptors for AGE

Parameters	Control C57BL/6 (n = 7–8)	Diabetic C57BL/6 (n = 7–8)	Control apo E-KO (n = 7–8)	Diabetic apo E-KO (n = 7–8)	Diabetic apo E-KO + Aminoguanidine (n = 7–8)	Diabetic apo E-KO + ALT-711 (n = 6–7)
AGE R-2 mRNA	1.30 ± 0.21	1.41 ± 0.39	1 ± 0.09	0.87 ± 0.08[d]	0.46 ± 0.05[f]	0.34 ± 0.04[g]
AGE R-3 mRNA	0.81 ± 0.44	2.24 ± 1.05[b]	1 ± 0.36	2.22 ± 0.31[c]	3.59 ± 1.34	2.47 ± 1.22
RAGE mRNA	1.02 ± 0.23	1.93 ± 0.73[b]	1 ± 0.19	1.46 ± 0.14	1.02 ± 0.07[f]	0.72 ± 0.11[g]
RAGE-positive staining (%)	0.42 ± 0.13	2.08 ± 0.47[c]	1.88 ± 0.37[c]	2.07 ± 0.47	0.62 ± 0.17[g]	0.93 ± 0.24[g]

[a] Data are mean ± SEM. AGE, advanced glycation end product; RAGE, receptor for advanced glycation end product.
[b] $P < 0.05$, [c] $P < 0.01$ *versus* appropriate nondiabetic control group.
[d] $P < 0.05$, [e] $P < 0.01$ *versus* appropriate C57BL/6 group.
[f] $P < 0.05$, [g] $P < 0.01$ *versus* diabetic apo E-KO mice.

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

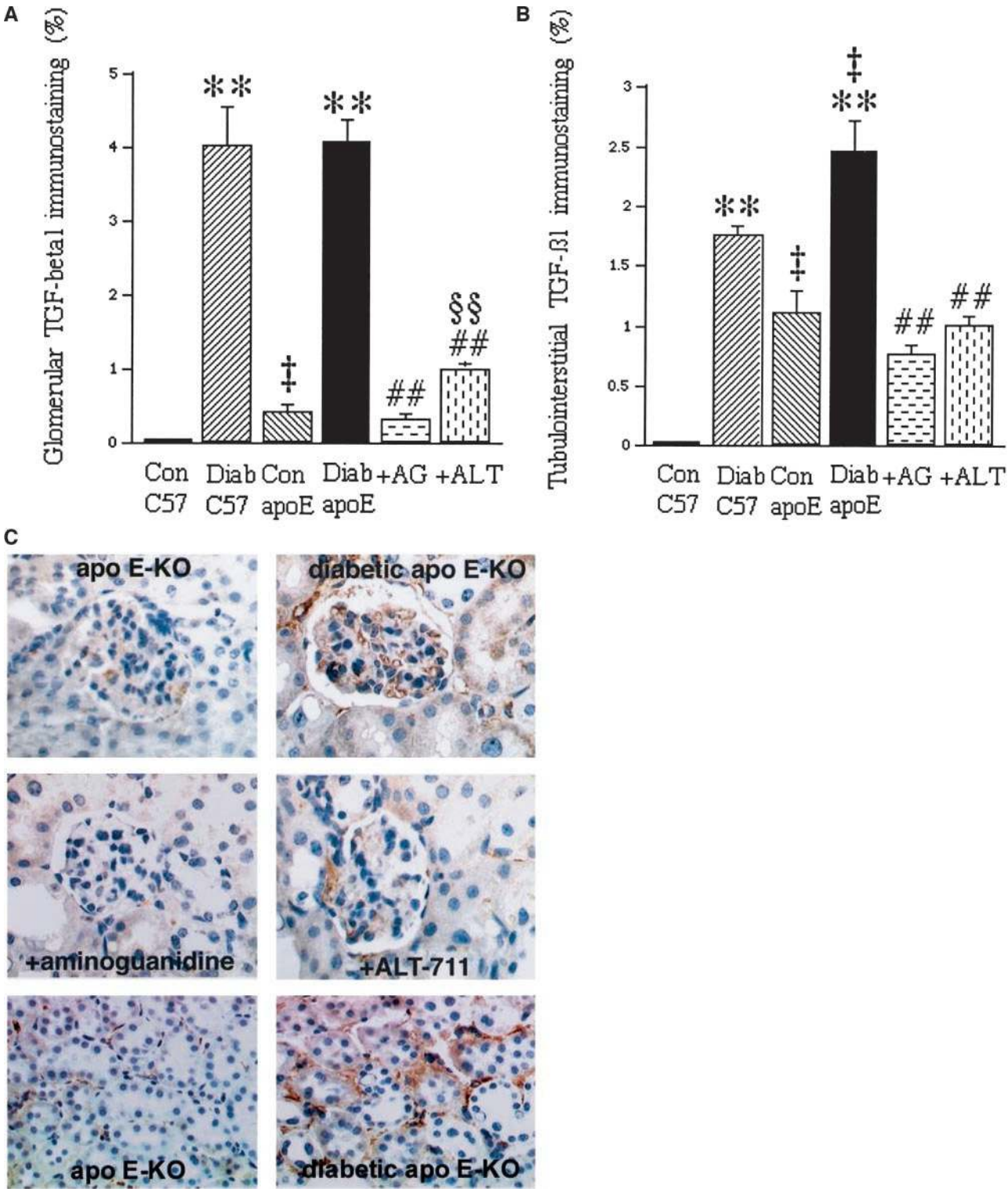

Figure 6. Quantification of TGF-β1 protein expression in the glomeruli (A) and the tubulointerstitium (B) as well as representative pictures of the kidneys (C; top, glomeruli; bottom, tubulointerstitium) after TGF-β1 immunostaining. Data are shown as mean ± SEM. $^{**}P < 0.01$ *versus* appropriate nondiabetic control group; $^{\ddagger}P < 0.01$ *versus* appropriate C57BL/6 group; $^{\#\#}P < 0.01$ *versus* diabetic apo E-KO mice; §§$P < 0.01$ *versus* diabetic apo E-KO mice treated with aminoguanidine. Magnification, ×400.

and lipid-dependent mechanisms (27). It is interesting that renal as well as circulating AGE were increased not only in diabetic animals but also in apo E-KO mice when compared with C57BL/6 mice both with and without diabetes. This suggests not only that the AGE in this model are derived from glucose-dependent pathways but also that lipids may contribute

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

to the accumulation of these AGE. Indeed, recent evidence has emphasized the importance of lipids as an important source of chemical modification of tissue proteins, even in the absence of hyperglycemia, leading to production of advanced lipoxidation products and consequently to renal injury (28). These chemically modified proteins include CML, which is also a major AGE. It is hypothesized that these AGE/advanced lipoxidation products, which accumulate in settings such as hyperlipidemia and chronic hyperglycemia, can induce renal injury by cross-link formation with collagens in the ECM, glomerular basement membrane, and tubular basement membrane or by inducing activation of a range of cellular processes *via* receptor-dependent pathways (28,29).

The renal injury in diabetic apo E-KO mice was associated with functional changes, including increased albuminuria and elevated serum urea and creatinine concentrations as well as various structural changes in both the glomerulus and the tubulointerstitium. Although diabetes *per se* in both mouse strains was associated with a range of abnormalities, including increased collagen accumulation and α-SMA expression, as well as functional and structural injury, not all of these parameters were increased in diabetic apo E-KO *versus* diabetic C57BL/6 mice. The major differences between the two strains with the induction of diabetes included increased albuminuria, glomerular injury index, tubulointerstitial injury, and tubular TGF-β1 immunostaining in the setting of a marked increase in renal AGE staining in these diabetic apo E-KO mice. The other major differences between the two diabetic strains, which had similar levels of glycemic control, were an increase in lipids and AGE in the diabetic apo E-KO mice. This suggests that AGE and lipids may play important roles in amplifying injury in the context of diabetes.

In the clinical context, the link between diabetic hyperlipidemia and renal damage has been postulated on the basis of a number of observations. For example, in subjects with diabetes with microalbuminuria or with overt proteinuria, plasma triglyceride levels are elevated compared with those without nephropathy (2). Furthermore, elevated serum cholesterol levels have been reported to be a predictor of rapid loss of renal function in patients with type 1 diabetes (3). The importance of hyperlipidemia in the development of diabetic nephropathy is further supported, albeit indirectly, by recent findings of a correlation between apo E gene polymorphisms and nephropathy in individuals with diabetes (30,31). However, the role of apo E polymorphism in diabetic nephropathy still remains controversial (32,33).

In addition to its effects on lipoprotein clearance, it has been suggested that apo E may confer protective renal effects independent of its effect on lipids (34). Mesangial cells are the major source of apo E in the kidneys, and apo E inhibits mesangial cell proliferation in an autocrine manner (34). Although the aggravated renal injury observed in diabetic apo E-KO mice in the present study most likely occurred as a result of the combination of diabetes and hyperlipidemia, these lipid-independent effects of apo E deficiency cannot be excluded in the progression of the disease.

The synergistic effects of hyperlipidemia and diabetes on the development of renal injury observed in the present study further extends our knowledge of the role of hyperlipidemia as an aggravating factor for diabetic nephropathy. Although diabetes was associated with renal damage in both C57BL/6 and apo E-KO mice, the hyperlipidemic animals with diabetes displayed a more extensive and severe degree of renal glomerular and tubulointerstitial injury as well as higher levels of urinary albumin excretion when compared with the normolipidemic diabetic animals. An earlier renal study using diabetic apo E-KO mice reported that relatively short-term diabetes (6 wk) resulted in increased renal expression of RAGE in association with infiltration of inflammatory cells (14). That study suggested that long-term diabetes might lead to accelerated renal disease in diabetic apo E-KO mice (14). Indeed, the present study has demonstrated that long-term diabetes leads to accelerated renal injury in diabetic apo E-KO mice.

The development of diabetic renal disease in hyperlipidemic animals in the current study is in agreement with studies using obese Zucker rats with diabetes (35). Because diabetic apo E-KO mice and Zucker rats are models of insulin deficiency and resistance, respectively, it is likely that the renal injury in these models is related to hyperglycemia *per se*.

The apo E-KO mouse is the most common experimental model currently used in the study of atherosclerosis. Renal pathology in this model has recently been characterized in detail, albeit in the absence of concomitant diabetes (4). As these mice get older, they develop mild progressive renal injury with spontaneous glomerular lesions with foam cells and widening of the mesangial area resembling the renal changes seen in human type III hyperlipoproteinemia (4). The present study has confirmed that hyperlipidemia alone does not cause significant renal impairment or major pathologic alterations in these animals, with only mild glomerular changes and negligible tubulointerstitial injury detected in the nondiabetic apo E-KO mice.

The interventional arm of the current study demonstrated that inhibitors of AGE accumulation conferred renoprotective effects, as assessed by a range of functional and structural parameters. These findings support the view that AGE play a pivotal role in the development and progression of renal injury in the diabetic apo E-KO mouse. In earlier studies, it was shown that soluble RAGE is able to confer renal protection in apo E-KO mice with short-term diabetes (14) as well as in genetically diabetic db/db mice (36). It needs to be appreciated that RAGE cannot be considered a highly specific AGE receptor because not only do AGE interact with other receptors, but also RAGE itself has multiple ligands, including S-100, which is itself a proinflammatory molecule (37). The present study provides further evidence to emphasize the role of advanced glycation and is consistent with the view that at least some of the renoprotective effects of approaches such as soluble RAGE are *via* effects on inhibiting AGE-dependent pathways.

The renoprotective effects of inhibition of AGE in experimental diabetic nephropathy have been previously described, albeit not in the context of hyperlipidemia. The prototype inhibitor of AGE formation, aminoguanidine, was shown to attenuate albuminuria and mesangial expansion in diabetic rats

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

(9). Because aminoguanidine is also an inhibitor of the inducible form of NO synthase (iNOS), subsequent studies using agents that are either iNOS inhibitors alone or more selective inhibitors of AGE formation (ALT-946) (38) that do not significantly affect NOS have also been examined. These studies have shown that the renoprotective effects of aminoguanidine relate to its AGE inhibitory action and not its ability to inhibit NOS (38). More recently, ALT-711 was shown to confer renoprotection in experimental type 1 diabetes (8) and in preliminary reports in the db/db mouse, a model of type 2 diabetes (39,40).

Increased renal accumulation of AGE in diabetic apo E-KO mice compared with nondiabetic apo E-KO mice was associated with overexpression of the prosclerotic growth factor TGF-β1. Furthermore, expression of TGF-β1 was ameliorated by treatment with either aminoguanidine or ALT-711. It is likely that the elaboration of TGF-β1 occurs partly as a result of AGE-induced expression of these proteins *via* receptor-dependent pathways (29). Indeed, both mesangial (12) and proximal tubular epithelial cells (13,41) have been demonstrated to express TGF-β1 upon exposure to AGE, which in turn induces rapid accumulation of ECM. Production of TGF-β1 may at least partly be in response to binding of AGE to their receptor RAGE (13). The effects of other AGE receptors have not been investigated as extensively, although the changes observed with respect to these receptors in response to both the diabetic milieu and the antiglycation therapies is consistent with these receptors' also playing an important role in the development of renal injury. The increase in renal AGE R3 expression observed in the present study in the diabetic kidney is in agreement with similar observations by our group in the diabetic rat heart (42).

Induction of diabetes in apo E-KO mice was associated with increased renal type I and IV collagen expression compared with nondiabetic apo E-KO mice. Blockade of the glycation pathway with aminoguanidine or ALT-711 was associated with a significant reduction in collagen I and IV immunostaining with a possible superiority of ALT-711 over aminoguanidine treatment. Previous studies by our group in a nonhyperlipidemic model of diabetic nephropathy have shown reduced expression of both type I and IV collagen with aminoguanidine or ALT-711 treatment (8,43). It is possible that the reduction in type I collagen expression is an important manifestation of the reduced tubulointerstitial injury seen with the antiglycation therapies. However, there was no difference in type I or IV collagen expression between diabetic C57BL/6 and diabetic apo E-KO mice despite the differences in renal structural and functional parameters, suggesting that the change in the expression of collagens is more closely linked with the diabetic milieu than to concomitant dyslipidemia.

Increased renal expression of α-SMA–positive cells in diabetic kidneys of C57BL/6 and apo E-KO mice observed in our study is in agreement with increasing evidence of the role of α-SMA–positive myofibroblasts, or activated fibroblasts, as well as activated mesangial cells in the pathogenesis of diabetic glomerulosclerosis and tubulointerstitial fibrosis (44,45). It has been shown that myofibroblast expression is markedly increased in areas of interstitial fibrosis (44), and this increased expression has been specifically demonstrated in human diabetic nephropathy (13).

ALT-711 treatment reduced expression of α-SMA–positive cells in both the glomeruli and the interstitium, whereas aminoguanidine had no effect on this parameter. This was an unexpected finding because both drugs had similar effect on AGE accumulation in both the kidney and the serum in the setting of renoprotection and reduced collagen deposition as well as attenuated TGF-β1 overexpression. The underlying explanation for this difference is unknown but may be related to the different mechanisms of action of these drugs. As mentioned previously, in addition to its principal action as an inhibitor of AGE formation, aminoguanidine has inhibitory action on NOS, particularly the inducible form (iNOS) (46). The effects of ALT-711 on α-SMA expression have been previously demonstrated by our group (13). Indeed, in those studies, it was shown that AGE *per se* promote tubulointerstitial transition as assessed by α-SMA immunostaining and that this phenomenon seems to act *via* RAGE- and TGF-β1–dependent pathways. The present study further extends these findings to the *in vivo* context linking AGE accumulation, RAGE, and TGF-β1 expression to the presence of α-SMA-positive cells, not only in the tubulointerstitium but also in the glomerulus.

It should be noted that not all molecular and cellular parameters assessed in this study were increased in diabetic apo E-KO mice when compared with diabetic C57BL/6 mice. This lack of effect of the addition of dyslipidemia to hyperglycemia on various parameters including macrophage infiltration and glomerular TGF-β1 and collagen expression is consistent with some of these pathways being more closely linked to glucose- rather than lipid-dependent pathways. Indeed, glucose promotes end-organ injury *via* a number of additional pathways to advanced glycation, such as activation of the renin-angiotensin system, induction of certain protein kinase C isoforms, promotion of mitochondrial oxidative stress, and increased flux *via* the hexosamine pathway (47,48). Although beyond the scope of this study, it is likely that some of these putative pathogenic mechanisms were induced by the diabetic milieu and amplify renal injury induced by AGE-dependent pathways.

Acknowledgments

This study was supported by a Centre Grant from the Juvenile Diabetes Research Foundation (JDRF). Markus Lassila was supported by grants from the Finnish Academy, Einar and Karin Stroem's Foundation, The Helsingin Sanomat Centennial Foundation, and Paavo Nurmi Foundation. Anna Calkin was supported by a scholarship from the National Heart Foundation; Wendy Burns by the Australian Kidney Foundation, and Merlin Thomas by a NHMRC Medical Postgraduate scholarship. Josephine Forbes is a JDRF Post-Doctoral Research Fellow. Terri Allen is a recipient of a Career Development Award jointly funded by the NHMRC and Diabetes Australia. Karin Jandeleit-Dahm is a clinical fellow of the National Heart Foundation of Australia.

The authors wish to thank Paula Aldersea, Gavin Langmaid, Maryann Arnstein, and Josefa Pete for excellent technical assistance. Reprinting of the pictures was supported by Alteon Inc.

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

References

1. Collins R, Armitage J, Parish S, Sleigh P, Peto R: MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: A randomised placebo-controlled trial. *Lancet* 361: 2005–2016, 2003
2. Hirano T: Lipoprotein abnormalities in diabetic nephropathy. *Kidney Int Suppl* 71: S22–S24, 1999
3. Krolewski AS, Warram JH, Christlieb AR: Hypercholesterolemia—A determinant of renal function loss and deaths in IDDM patients with nephropathy. *Kidney Int Suppl* 45: S125–S131, 1994
4. Wen M, Segerer S, Dantas M, Brown PA, Hudkins KL, Goodpaster T, Kirk E, LeBoeuf RC, Alpers CE: Renal injury in apolipoprotein E-deficient mice. *Lab Invest* 82: 999–1006, 2002
5. Sugimoto K, Tsuruoka S, Fujimura A: Effect of enalapril on diabetic nephropathy in OLETF rats: The role of an anti-oxidative action in its protective properties. *Clin Exp Pharmacol Physiol* 28: 826–830, 2001
6. Fried LF, Orchard TJ, Kasiske BL: Effect of lipid reduction on the progression of renal disease: a meta-analysis. *Kidney Int* 59: 260–269, 2001
7. Jandeleit-Dahm K, Cao Z, Cox AJ, Kelly DJ, Gilbert RE, Cooper ME: Role of hyperlipidemia in progressive renal disease: Focus on diabetic nephropathy. *Kidney Int Suppl* 71: S31–S36, 1999
8. Forbes JM, Thallas V, Thomas MC, Founds HW, Burns WC, Jerums G, Cooper ME: The breakdown of preexisting advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. *FASEB J* 17: 1762–1764, 2003
9. Soulis-Liparota T, Cooper M, Papazoglou D, Clarke B, Jerums G: Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes* 40: 1328–1334, 1991
10. Sell DR, Lapolla A, Odetti P, Fogarty J, Monnier VM: Pentosidine formation in skin correlates with severity of complications in individuals with long-standing IDDM. *Diabetes* 41: 1286–1292, 1992
11. Nakamura S, Tachikawa T, Tobita K, Aoyama I, Takayama F, Enomoto A, Niwa T: An inhibitor of advanced glycation end product formation reduces N epsilon-(carboxymethyl)lysine accumulation in glomeruli of diabetic rats. *Am J Kidney Dis* 41: S68–S71, 2003
12. Throckmorton DC, Brogden AP, Min B, Rasmussen H, Kashgarian M: PDGF and TGF-beta mediate collagen production by mesangial cells exposed to advanced glycosylation end products. *Kidney Int* 48: 111–117, 1995
13. Oldfield MD, Bach LA, Forbes JM, Nikolic-Paterson D, McRobert A, Thallas V, Atkins RC, Osicka T, Jerums G, Cooper ME: Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). *J Clin Invest* 108: 1853–1863, 2001
14. Kislinger T, Tanji N, Wendt T, Qu W, Lu Y, Ferran LJ Jr, Taguchi A, Olson K, Bucciarelli L, Goova M, Hofmann MA, Cataldegirmen G, D'Agati V, Pischetsrieder M, Stern DM, Schmidt AM: Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 21: 905–910, 2001
15. Wolffenbuttel 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
16. Candido R, Jandeleit-Dahm KA, Cao Z, Nesteroff SP, Burns WC, Twigg SM, Dilley 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. 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
18. Allen TJ, Cooper ME, O'Brien RC, Bach LA, Jackson B, Jerums G: Glomerular filtration rate in streptozocin-induced diabetic rats. Role of exchangeable sodium, vasoactive hormones, and insulin therapy. *Diabetes* 39: 1182–1190, 1990
19. Cao Z, Bonnet B, Candido R, Nesteroff S, Burns M, Kawachi H, Shimizu F, Carey R, de Gasparo M, Cooper M: Angiotensin type 2 receptor antagonism confers renal protection in a rat model of progressive renal injury. *J Am Soc Nephrol* 13: 1773–1787, 2002
20. Kelly DJ, Wilkinsonberka JL, Allen TJ, Cooper ME, Skinner SL: A new model of diabetic nephropathy with progressive renal impairment in the transgenic (Mren-2)27 rat (Tgr). *Kidney Int* 54: 343–352, 1998
21. Hume DA, Gordon S: Mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Identification of resident macrophages in renal medullary and cortical interstitium and the juxtaglomerular complex. *J Exp Med* 157: 1704–1709, 1983
22. 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
23. 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
24. Bonnet F, Cooper ME, Carey RM, Casley D, Cao Z: Vascular expression of angiotensin type 2 receptor in the adult rat: influence of angiotensin II infusion. *J Hypertens* 19: 1075–1081, 2001
25. Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD: Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. *J Am Soc Nephrol* 11: 1656–1666, 2000
26. Miura J, Yamagishi S, Uchigata Y, Takeuchi M, Yamamoto H, Makita Z, Iwamoto Y: Serum levels of non-carboxymethyllysine advanced glycation endproducts are correlated to severity of microvascular complications in patients with type 1 diabetes. *J Diabetes Complications* 17: 16–21, 2003
27. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR: The advanced glycation end product, Nϵ-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem* 271: 9982–9986, 1996
28. 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
29. Vlassara H, Palace MR: Diabetes and advanced glycation endproducts. *J Intern Med* 251: 87–101, 2002
30. Araki S, Moczulski DK, Hanna L, Scott LJ, Warram JH, Krolewski AS: APOE polymorphisms and the development of

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

diabetic nephropathy in type 1 diabetes: Results of case-control and family-based studies. *Diabetes* 49: 2190–2195, 2000
31. Eto M, Saito M, Okada M, Kume Y, Kawasaki F, Matsuda M, Yoneda M, Matsuki M, Takigami S, Kaku K: Apolipoprotein E genetic polymorphism, remnant lipoproteins, and nephropathy in type 2 diabetic patients. *Am J Kidney Dis* 40: 243–251, 2002
32. Onuma T, Laffel LM, Angelico MC, Krolewski AS: Apolipoprotein E genotypes and risk of diabetic nephropathy. *J Am Soc Nephrol* 7: 1075–1078, 1996
33. Ukkola O, Kervinen K, Salmela PI, von Dickhoff K, Laakso M, Kesaniemi YA: Apolipoprotein E phenotype is related to macro- and microangiopathy in patients with non-insulin-dependent diabetes mellitus. *Atherosclerosis* 101: 9–15, 1993
34. Chen G, Paka L, Kako Y, Singhal P, Duan W, Pillarisetti S: A protective role for kidney apolipoprotein E. Regulation of mesangial cell proliferation and matrix expansion. *J Biol Chem* 276: 49142–49147, 2001
35. Coimbra TM, Janssen U, Grone HJ, Ostendorf T, Kunter U, Schmidt H, Brabant G, Floege J: Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes. *Kidney Int* 57: 167–182, 2000
36. 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
37. 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
38. Soulis T, Cooper ME, Sastra S, Thallas V, Panagiotopoulos S, Bjerrum OJ, Jerums G: Relative contributions of advanced glycation and nitric oxide synthase inhibition to aminoguanidine-mediated renoprotection in diabetic rats. *Diabetologia* 40: 1141–1151, 1997
39. Peppa M, Brem H, Chai W, Basgen J, Lu M, Zhu L, Steffes M, Vlassara H: AGE-breaker, ALT-711 improves established diabetic nephropathy in db/db mice. *Diabetes* 52: A189, 2003
40. Kwon MK, Ha H, Kim WY, Kim J, Nagai R, Horiuchi S, Lee HB: ALT-711, an "AGE cross-link breaker," ameliorates renal injury in db/db mice. *Nephrol Dial Transplant* 18: M154, 2003
41. Gilbert RE, Cox A, Wu LL, Allen TJ, Hulthen UL, Jerums G, Cooper ME: Expression of transforming growth factor-β1 and type IV collagen in the renal tubulointerstitium in experimental diabetes: Effects of ACE inhibition. *Diabetes* 47: 414–422, 1998
42. 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
43. Kelly DJ, Gilbert RE, Cox AJ, Soulis T, Jerums G, Cooper ME: Aminoguanidine ameliorates overexpression of prosclerotic growth factors and collagen deposition in experimental diabetic nephropathy. *J Am Soc Nephrol* 12: 2098–2107, 2001
44. Pedagogos E, Hewitson T, Fraser I, Nicholls K, Becker G: Myofibroblasts and arteriolar sclerosis in human diabetic nephropathy. *Am J Kidney Dis* 29: 912–918, 1997
45. Essawy M, Soylemezoglu O, Muchaneta-Kubara EC, Shortland J, Brown CB, el Nahas AM: Myofibroblasts and the progression of diabetic nephropathy. *Nephrol Dial Transplant* 12: 43–50, 1997
46. Tilton RG, Chang K, Hasan KS, Smith SR, Petrash JM, Misko TP, Moore WM, Currie MG, Corbett JA, McDaniel ML, *et al.*: Prevention of diabetic vascular dysfunction by guanidines. Inhibition of nitric oxide synthase versus advanced glycation end-product formation. *Diabetes* 42: 221–232, 1993
47. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813–820, 2001
48. Sheetz MJ, King GL: Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 288: 2579–2588, 2002

Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.