

# Reduction of the Accumulation of Advanced Glycation End Products by ACE Inhibition in Experimental Diabetic Nephropathy

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The effect of ACE inhibition on the formation of advanced glycation end products (AGEs) and oxidative stress was explored. Streptozocin-induced diabetic animals were randomized to no treatment, the ACE inhibitor ramipril (3 mg/l), or the AGE formation inhibitor aminoguanidine (1 g/l) and followed for 12 weeks. Control groups were followed concurrently. Renal AGE accumulation, as determined by immunohistochemistry and both serum and renal fluorescence, were increased in diabetic animals. This was attenuated by both ramipril and aminoguanidine to a similar degree. Nitrotyrosine, a marker of protein oxidation, also followed a similar pattern. The receptor for AGEs, gene expression of the membrane-bound NADPH oxidase subunit gp91phox, and nuclear transcription factor- $\kappa$ B were all increased by diabetes but remained unaffected by either treatment regimen. Two other AGE receptors, AGE R2 and AGE R3, remained unchanged for the duration of the study. The present study has identified a relationship between the renin-angiotensin system and the accumulation of AGEs in experimental diabetic nephropathy that may be linked through oxidative stress *Diabetes* 51:3274–3282, 2002

**D** diabetes is characterized by chronic hyperglycemia, which facilitates nonenzymatic browning in the Maillard reaction between reducing sugars and free reactive amino groups of proteins (1). Subsequent biochemical modifications result in the irreversible formation of a heterogeneous group of products known as advanced glycation end products (AGEs). Their importance in the pathogenesis of diabetes was first

recognized in studies investigating the effects of the advanced glycation inhibitor aminoguanidine on vascular wall cross-linking (2) and on various aspects of experimental diabetic nephropathy where significant improvements in both renal functional and structural parameters were observed (3).

Oxidative stress is widely recognized as a key component in the development of diabetes complications (4,5). It is unknown, however, whether it is an important early link between hyperglycemia and complications or whether it is a consequence of primary pathogenic mechanisms (6). AGEs have long been associated with increased oxidative stress both in vitro (7,8) and in vivo (9,10). A recent study has demonstrated that inhibition of reactive oxygen species (ROS) in cultured bovine endothelial cells exposed to glucose interferes with multiple independent pathways of hyperglycemic damage, including nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation and formation of AGEs (5). Furthermore, a recent study has demonstrated activation of NADPH oxidase by AGEs (8).

Interruption of the renin-angiotensin system (RAS) with drugs such as ACE inhibitors (ACEIs) has proven beneficial in diabetic renal disease (11). Multiple mechanisms have been postulated to explain the renoprotective effects of these agents. It is conceivable that blockade of the RAS alone may have effects on AGE accumulation by either improving renal function or reducing oxidative stress. Culture experiments using NRK-49F cells exposed to AGEs demonstrated a reversal in AGE-induced collagen production by the ACEI captopril (12). In addition, in that study, increased protein expression of the receptor for AGEs (RAGE) was also attenuated by ACE inhibition. In vivo studies with the AII receptor antagonist losartan in a model of renal ablation showed decreases in serum AGE concentrations and improvements in renal function independent of changes in the profibrotic cytokine transforming growth factor  $\beta$  (13). Losartan also attenuated increases in free radical production in angiotensin II-associated hypertension (14).

This study investigates the effects of blockade of the RAS by ramipril and its relationship to advanced glycation in experimental diabetic nephropathy. Markers of oxidative stress and NF- $\kappa$ B activation were also examined since both of these parameters have been reported to be modulated by angiotensin II and AGEs.

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ACEI, ACE inhibitor; AER, albumin excretion rate; AGE, advanced glycation end product; CML, carboxymethyllysine; EMSA, electrophoretic mobility shift assay; GFR, glomerular filtration rate; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; RAS, renin-angiotensin system; RAGE, receptor for AGEs; ROS, reactive oxygen species; rRNA, ribosomal RNA.

## RESEARCH DESIGN AND METHODS

**Experimental animal model.** Experimental diabetes was induced in male Sprague Dawley rats (200–250 g) by injection of streptozocin (50 mg/kg) after an overnight fast. Animals with plasma glucose concentration >15 mmol/l 1 week postinduction of diabetes were included in the study as diabetic. Sham-injected control animals (sodium citrate buffer pH 4.5) were followed concurrently. Diabetic and control animals were randomized into groups ( $n = 6$ ) that received 1) no treatment, 2) ramipril (Hoechst, Frankfurt, Germany) at 3 mg/l for diabetic rats and 9 mg/l for control rats in drinking water, or 3) aminoguanidine (Fluka Chemica, Buchs, Switzerland) at 1 g/l for diabetic and 3 g/l for control rats in drinking water for 12 weeks. Two units of Ultralente insulin (Ultratard HM; Novo Industries, Bagsvaerd, Denmark) were administered daily to diabetic animals to prevent ketoacidosis and improve survival. Body weight, glomerular filtration rate (GFR), mean systolic blood pressure by tail cuff plethysmography (15), albumin excretion rate (AER) (16), and HbA<sub>1c</sub> (17) were measured every 4 weeks. All animal procedures were in accordance with guidelines set by the Austin Hospital Ethics Committee and the National Health and Medical Research Council of Australia.

**Immunohistochemistry.** A modification of the ABC immunoglobulin enzyme bridge technique (18) was used for immunohistochemistry. Formalin-fixed paraffin sections of kidney were dewaxed and hydrated. After incubation with 0.3% hydrogen peroxide for 20 min, sections were incubated with protein-blocking agent (Lipshaw, Pittsburgh, PA) for AGE/RAGE or normal swine serum for nitrotyrosine for 20 min and then primary antibody for 1 h. The polyclonal AGE antibody used in this protocol (1:500) has been described in detail previously and recognizes primarily carboxymethyllysine (CML) (19). Other primary antibodies used were polyclonal goat anti-RAGE (1:250) (Chemicon, Temecula, CA) and rabbit anti-nitrotyrosine (1:120) (Upstate Biotechnology, Lake Placid, NY) (20).

Tissue sections were consecutively stained 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 (DAB; Sigma Chemical, St Louis, MO) 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. Quantitation of renal cortical AGE, RAGE, and nitrotyrosine immunostaining was completed by computer-aided densitometry (MCID Video Pro-32; MCID, Bedford Park, SA, Australia), where a total of 20 fields ( $\times 100$ ) were counted per section and six animals per group were counted. Results were expressed as proportional area of positive staining (21).

**Western immunoblotting for nitrotyrosine.** Kidney samples were homogenized in neutral salt buffer containing protease inhibitors (50 mmol/l Tris-Cl, pH 7.4, 150 mmol/l NaCl, 5 mmol/l EDTA, 10  $\mu$ g/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and aprotinin 1  $\mu$ g/ml). Cell debris was pelleted by centrifugation at 400g and the supernatant further separated by ultracentrifugation at 75,000g. The protein content of the second supernatant was determined using the Bicinchoninic method (Pierce, Rockford, IL) and 5  $\mu$ g separated by 10% SDS-PAGE at 200V for 1 h (Biorad Laboratories, Richmond, CA). After wet transfer at 100V for 1 h onto nitrocellulose membrane, the filter was blocked with 5% BLOTTO (5% skim milk, 1 $\times$  Tris-buffered saline, 0.05% Tween 20) for 1 h. Monoclonal mouse anti-nitrotyrosine (1:15,000) (Upstate Biotechnologies, Lake Placid, NY) was added and the filter incubated overnight at 4°C with gentle rocking. After washing (1 $\times$  Tris-buffered saline, 0.05% Tween 20), sequential amplification was performed with biotinylated rabbit anti-mouse (1:15,000) (Dako, Carpinteria, Denmark) and then horseradish peroxidase-conjugated streptavidin (1:20,000) (Dako). Immunoreactivity was determined by use of an ECL Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and light emission captured on X-ray film for 5 min (Kodak, Rochester, NY). Band thickness at 66 kDa was determined by computer-aided densitometry (Video Pro-32).

**Fluorescence for tissue and serum AGEs.** As described previously (22), collagen was extracted from kidney samples. The resulting supernatant was normalized to 1 mg/ml of protein (BCA protein assay; Pierce) and used to measure collagen-related fluorescence with excitation/emission at 370/440 nm (2). The fluorescence of an enzyme blank (type IV collagenase and proteinase K) was subtracted from the tissue fluorescence measurements as previously described (23).

Fluorescent AGE peptides present in serum were assayed using on-line spectrofluorometric detection in a flow system (24). Briefly, 20  $\mu$ l serum was added to 480  $\mu$ l of 0.15 mmol/l trichloroacetic acid and 100  $\mu$ l chloroform. Samples were shaken vigorously, centrifuged, and 20  $\mu$ l of the aqueous layer was then injected into a flow system using a Waters sample injector and high-pressure liquid chromatography. 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. Results are expressed in units per milliliter with adjustment for peptide content.

**Real-time PCR.** cDNA was synthesized from RNA extracted from kidneys with the Superscript First Strand synthesis system (Gibco BRL, Grand Island, NY). Gene expression was analyzed by real-time quantitative RT-PCR, performed with the TaqMan system as previously described (25). In brief, this system is based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Foster City, CA). Fluorescence for each cycle was quantitatively analyzed (Perkin-Elmer, PE Biosystems). Gene expression of the target sequence was normalized in relation to the endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700). Each probe was optimized individually for the renal samples studied. Primers and Taqman probes for target genes and 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700). RAGE-F: CCCTGACCT GTGCCATCTCT R: GGGTGTGCCATCTTTTATCCA P: 6FAM-CCCAGCCTC CCCCTCAAATCCA-TAMRA. AGER2-F: CGAGCTCACCACCAATGAGT R: TTTGGGTTTCTGGGAGACCA P: 6FAM-CCGTACTCCGGCTTTGCCCTTCA-TAMRA. AGER3-F: GACGGCTTCTCACTTAATGATGC R: CATGCACCAGGC CATCT P: 6FAM-TTAGCTGGCTCTGAAACCCAAACCCT-TAMRA. NADPH gp91phox-F: CAA GGT TTA TGA CGA TGA GCC TAA R: CCG ACA CAC TGG CAG CAA P: 6FAM-CACCTGCAGCCTGCCTGAATTCA-TAMRA.

cDNA, which was synthesized from tissue-extracted RNA, underwent the following amplification protocol: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 94°C for 20 s and 60°C for 1 min. Each sample was tested in triplicate and two separate assays performed for each probe on different days. Results were expressed relative to control kidney values, which were arbitrarily assigned a value of 1. Intra- and interassay variation were also assessed.

### Electrophoretic mobility shift assay

**Preparation of nuclear extracts.** The following is based on the method of Morrissey et al. (26) with minor modifications. Frozen kidney cortex was weighed, minced (~100 mg), and placed in 10 volumes of cold Buffer A (10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 0.1 mmol/l dithiothreitol, and 1 mmol/l PMSF). IGEAL (Sigma Chemical) was added to a final concentration of 0.7%. The homogenates were centrifuged at 7,000g for 30 s and resuspended in 1 ml fresh buffer A. After centrifugation, the samples were resuspended in 0.3 ml buffer B (20 mmol/l HEPES, pH 7.9, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, and 1 mmol/l PMSF) and left on ice for 20 min. Finally, the samples were centrifuged at 10,000g for 30 min and the supernatant removed. Protein concentration was determined by the Bicinchoninic method (Pierce).

**Electromobility shift assay.** Double-stranded NF- $\kappa$ B consensus oligonucleotides (5'-AGTTGAGGGGACTTTCCAGG-3'; Promega, Madison, WI) were end labeled with  $\gamma$ -<sup>32</sup>P-ATP (GeneWorks Pty, Adelaide, SA, Australia) using T4 kinase (Promega) and T4 kinase buffer (Promega). The binding of nuclear protein to the radiolabelled oligonucleotide was performed by equilibrating 7  $\mu$ g nuclear protein extract, 0.25  $\mu$ l poly dIdC (Amersham Pharmacia Biotech), and 10  $\mu$ l binding buffer (5 mmol/l MgCl<sub>2</sub>, 5 mmol/l Tris-HCl, 20% glycerol, 2.5 mmol/l EDTA, and 2.5 mmol/l dithiothreitol) to a total volume of 19  $\mu$ l in distilled water. This was followed by the addition of 1  $\mu$ l of <sup>32</sup>P-labeled NF- $\kappa$ B probe (150,000 cpm, Cherenkov counting) and incubated for 15 min. The DNA-protein complexes were resolved by nondenaturing electrophoresis on a 7% polyacrylamide gel at 150V for 50 min, followed by autoradiography (Kodak Biomax, Rochester, NY) with intensifying screens for 7–12 h at -70°C.

The specificity of the NF- $\kappa$ B electrophoretic mobility shift assay (EMSA) was determined in 100-fold excess of either unlabelled NF- $\kappa$ B oligonucleotide or mutant NF- $\kappa$ B oligonucleotide (5'-AGTTGAGGCGACTTTCCAGG-3'; Santa Cruz Biotechnology, Santa Cruz, CA). These were added to the nuclear protein binding reaction 15 min before the labeled NF- $\kappa$ B probe and EMSA performed as described above.

**Statistical analysis.** Results are expressed as means  $\pm$  SE, unless otherwise specified. Data for albuminuria were not normally distributed and therefore analyzed after logarithmic transformation. Analyses were performed by ANOVA followed by post hoc analysis using Fisher's least significant difference method, correcting for multiple comparisons (17).  $P < 0.05$  was considered statistically significant.

## RESULTS

**Biochemical and metabolic parameters.** At 12 weeks, diabetic animals had a significant increase in blood glucose and HbA<sub>1c</sub> levels as compared with nondiabetic control groups ( $P < 0.005$  vs. control). However, no differences in glycemic control were noted between

TABLE 1  
Biochemical and metabolic parameters for all animals at week 12

	Control	Control plus ACEI	Control plus AG	Diabetic	Diabetic plus ACEI	Diabetic plus AG
<i>n</i>	6	6	6	6	6	6
Glucose (mmol/l)	5.4 ± 0.2	5.8 ± 0.2	5.6 ± 0.2	22.1 ± 1.7*	18.4 ± 2.0*	21.4 ± 1.5*
HbA <sub>1c</sub> (%)	3.6 ± 0.2	3.3 ± 0.6	3.9 ± 0.2	10.6 ± 0.4*	9.1 ± 0.7*	9.7 ± 0.5*
Body weight (g)	453 ± 8	402 ± 17	420 ± 12	302 ± 15*	314 ± 22*	341 ± 22*
Kidney-to-body weight ratio (×10 <sup>-3</sup> )	6.4 ± 0.3	7.1 ± 0.2	6.6 ± 0.1	10.7 ± 0.4*	10.0 ± 0.0*	10.7 ± 0.0*
GFR (ml/min)	3.2 ± 0.2	3.3 ± 0.2	3.2 ± 0.1	3.9 ± 0.2†	3.5 ± 0.3	4.1 ± 0.2*
Urine volume (ml/24 h)	15 ± 1	22 ± 2	25 ± 2	121 ± 12*	76 ± 14*	107 ± 16*
Blood pressure (mmHg)	133 ± 3	130 ± 7	140 ± 5	147 ± 3	129 ± 7‡	144 ± 5
Serum AGEs	8.7 ± 0.6	8.3 ± 0.6	7.7 ± 0.6	11.6 ± 0.4*	8.3 ± 0.6‡	8.7 ± 0.5‡

Data are means ± SE. \**P* < 0.005; †*P* < 0.02 vs. appropriate control; ‡*P* < 0.05 vs. diabetic. AG, aminoguanidine.

treated and untreated diabetic groups (Table 1). Diabetic animals also had decreased body weights but large increases in kidney-to-body weight ratio, indicating renal hypertrophy. No attenuation of these changes was demonstrated with aminoguanidine or ACEI treatments (Table 1). Control rats treated with aminoguanidine or ramipril did not show any differences from the untreated control groups with respect to GFR or urine volume. Diabetic animals had significant increases in GFR and urine volume when compared with controls, but no effects on either parameter were noted with any of the treatment regimens (Table 1). Systolic blood pressure was significantly reduced in diabetic rats receiving ramipril as compared with untreated diabetic animals. No differences were observed in systolic blood pressure in diabetic animals treated with aminoguanidine (Table 1).

Diabetes was associated with a significant increase in AER at 12 weeks, which was attenuated by treatment with ramipril and to a lesser extent with aminoguanidine (Fig. 1). Albumin excretion was unchanged by the administration of ramipril or aminoguanidine in control rats.

**Quantitation of AGEs.** Total renal fluorescence was increased in the diabetic group when compared with the control group (Fig. 2A). ACE inhibition was associated with reduced renal fluorescence in both the control and diabetic treatment groups as compared with their untreated counterparts. The aminoguanidine-treated groups

also showed a decrease in renal fluorescence in both control and diabetic treatment groups. Serum levels of fluorescent AGEs were found to be increased in the diabetic rats (Table 1). Aminoguanidine and ramipril were equally effective in reducing these diabetes-induced increases in serum AGE peptides. Serum AGE levels in control animals treated with aminoguanidine were also decreased when compared with control alone. Immunohistochemical staining of AGEs in the cortex of the kidney demonstrated a significant increase in the diabetic as compared with the control group. This was attenuated by

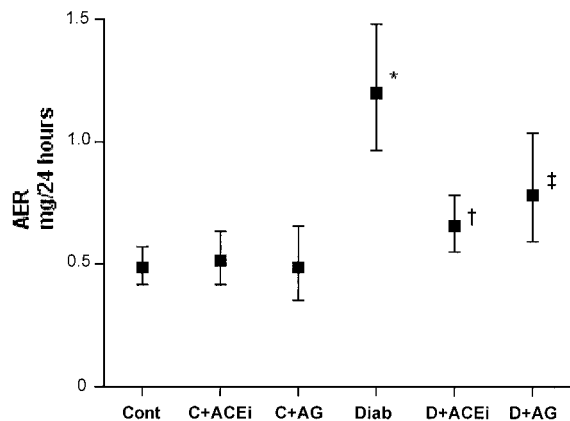


FIG. 1. Albumin excretion over 24 h at week 12. Data are expressed as geometric mean ×/÷ tolerance factor. \**P* < 0.005 vs. control; †*P* < 0.001, ‡*P* < 0.05 vs. diabetic. Cont, control; C+ACEi, control plus ACEI; C+AG, control plus aminoguanidine; Diab, diabetic; D+ACEi, diabetic plus ACEI; D+AG, diabetic plus aminoguanidine.

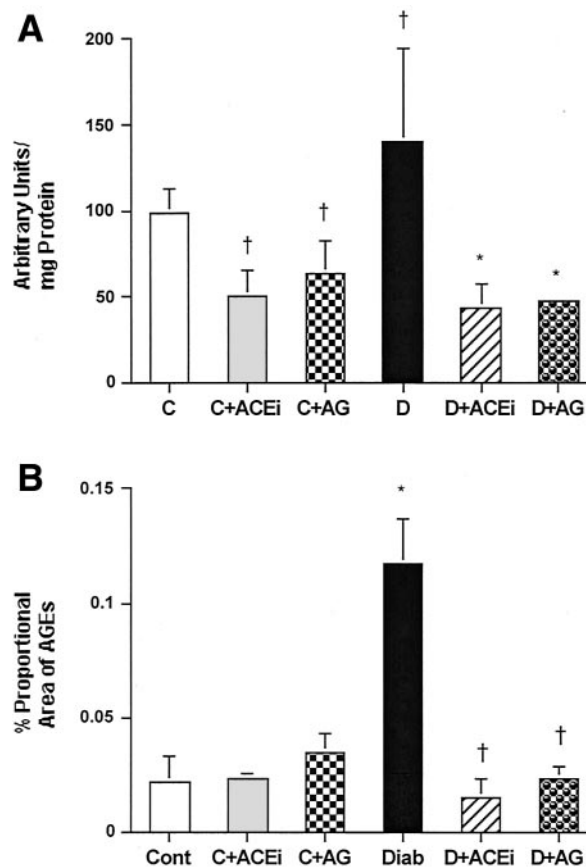


FIG. 2. A: Renal AGE fluorescence at 370/440 nm corrected for protein content. \**P* < 0.001 vs. diabetic; †*P* < 0.05 vs. control. B: Morphometric analysis of immunostaining for renal AGEs. \**P* < 0.001 for control vs. diabetic; †*P* < 0.01 vs. diabetic. C, control; C+ACEi, control plus ACEI; C+AG, control plus aminoguanidine; D, diabetic; D+ACEi, diabetic plus ACEI; D+AG, diabetic plus aminoguanidine.

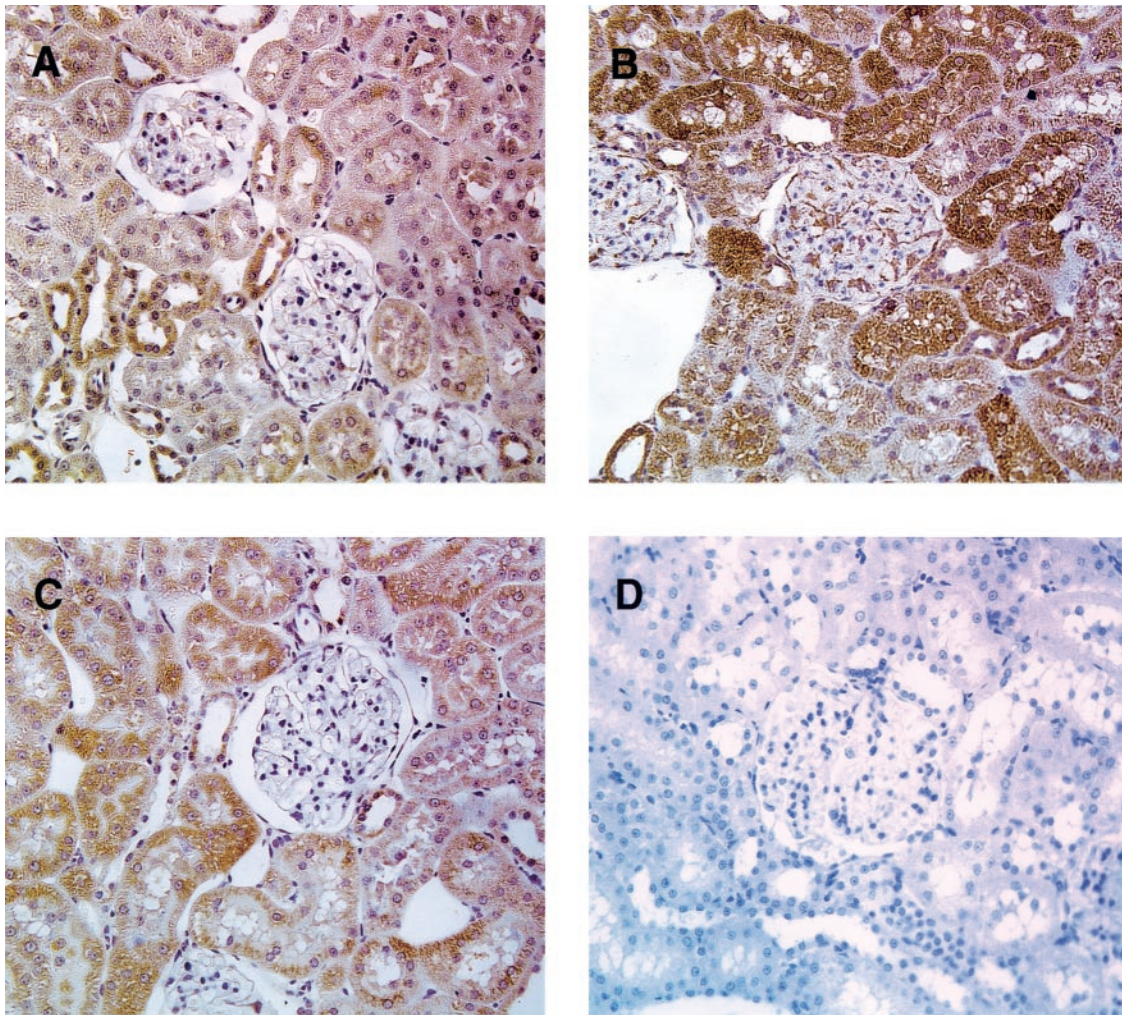


FIG. 3. Immunohistochemical staining for renal AGEs at week 12. *A*: Control kidney; *B*: Diabetic kidney; *C*: Diabetic kidney plus ACE inhibition; *D*: negative control kidney. Magnification  $\times 400$ .

both aminoguanidine and ramipril (Fig. 2*B*, Fig. 3). The changes in the amount of staining appeared most evident in the glomerulus, but there were also significant changes in the tubulointerstitium.

**AGE receptor quantitation.** The proportional area of RAGE immunostaining was increased in all diabetic animals as compared with the control group (Fig. 4*A*). RAGE in the diabetic groups was localized to proximal tubules and glomeruli. Neither ramipril nor aminoguanidine significantly reduced the amount of RAGE; however, there was a trend toward less RAGE immunostaining, particularly in the advanced glycation-treated group.

Real-time RT-PCR demonstrated no significant difference in renal RAGE gene expression between the control and diabetic groups (control  $1.02 \pm 0.1$ , diabetic  $0.87 \pm 0.09$  arbitrary units;  $P = 0.57$ ). No other changes were observed among groups, although a modest decrease in RAGE gene expression was observed in the control group treated with ramipril as compared with the diabetic group treated with ramipril (control plus ACEI  $0.57 \pm 0.06$ , diabetic plus ACEI  $1.25 \pm 0.29$ ;  $P < 0.05$ ). The interassay coefficient of variation was 2.5% and the intra-assay coefficient of variation was 1.3% for RAGE gene expression by RT-PCR.

Changes in AGE R2 gene expression in the kidney

paralleled those seen in RAGE mRNA levels. There were no differences observed between control and diabetic animals (control  $1.0 \pm 0.1$ , diabetic  $0.8 \pm 0.1$ ;  $P = 0.22$ ). There was, however, a significant difference noted between the control and diabetic groups treated with the ACEI (control plus ACEI  $0.57 \pm 0.12$ , diabetic plus ACEI

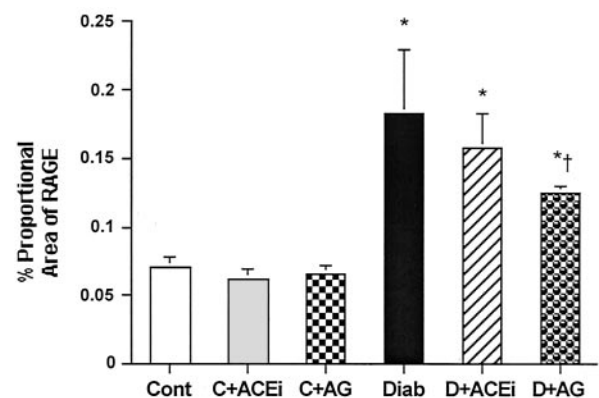


FIG. 4. Morphometric analysis of immunostaining for renal RAGE at week 12. \* $P < 0.001$  vs. control; † $P = 0.07$  vs. diabetic. Cont, control; C+ACEi, control plus ACEI; C+AG, control plus aminoguanidine; Diab, diabetic; D+ACEi, diabetic plus ACEI; D+AG, diabetic plus aminoguanidine.

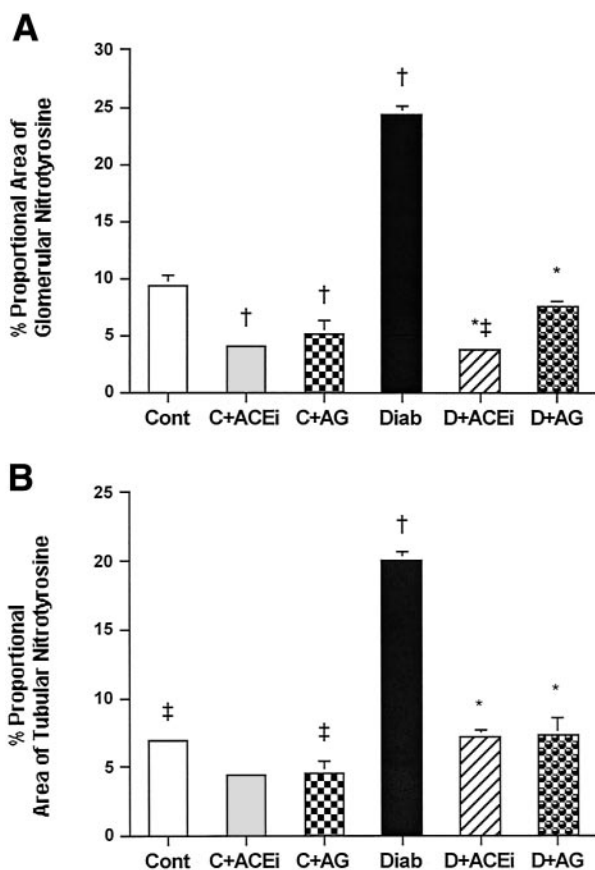


FIG. 5. A: Morphometric analysis of immunostaining for glomerular nitrotyrosine at week 12. \* $P < 0.005$  vs. control; † $P < 0.001$  vs. diabetic. B: Morphometric analysis of immunostaining for tubular nitrotyrosine at week 12. \* $P < 0.001$  vs. diabetic; † $P < 0.001$  vs. control; ‡ $P < 0.01$  vs. control plus ACEI. Cont, control; C+ACEI, control plus ACEI; C+AG, control plus aminoguanidine; Diab, diabetic; D+ACEI, diabetic plus ACEI; D+AG, diabetic plus aminoguanidine.

$1.09 \pm 0.13$ ;  $P < 0.05$ ). The interassay variation for AGE R2 was 0.9%, with an intra-assay sample variation of 0.09%.

Renal expression of the AGE R3 gene demonstrated no significant differences among the various groups. There was however a tendency for AGE R3 gene expression in the groups treated with ACEIs to be lower than in the other groups. The interassay variation was 1.6% for AGE R3, with an intra-assay variation within a single assay at 0.55%.

**Nitrotyrosine and gp91phox subunit of NADPH oxidase.** Nitrotyrosine formation was used as an index of damage related to reactive nitrogen species. Increased immunostaining for nitrotyrosine was observed in the glomeruli (control  $9.4 \pm 0.9$ , diabetic  $24.3 \pm 0.7\%$ ;  $P < 0.001$ ) (Fig. 5A) and tubules (control  $6.9 \pm 0.3$ , diabetic  $20.0 \pm 0.6\%$ ;  $P < 0.001$ ) (Fig. 5B) of diabetic animals (Fig. 6A and B). These results were confirmed by Western blotting (control  $193.6 \pm 20.2$ , diabetic  $281.4 \pm 18.7$  DxA;  $P < 0.001$ ). Nitrotyrosine was mostly localized to the apical region of proximal tubule cells and in the glomeruli. This increased staining was attenuated by treatment with ramipril (glomerular diabetic plus ACEI  $3.8 \pm 0.2\%$ ,  $P < 0.001$ ; tubule diabetic plus ACEI  $7.2 \pm 0.4$ ,  $P < 0.001$ ) and aminoguanidine (glomerular diabetic plus advanced glycation  $7.5 \pm 0.5\%$ ,  $P < 0.001$ ; tubule diabetic plus advanced glycation  $8.3 \pm 1.2\%$ ,  $P < 0.001$ ) (Fig. 5A and B, Fig. 6C and D). A reduction in total nitrotyrosine was also demon-

strated by Western blotting (diabetic plus ACEI  $137.3 \pm 21.2$ , diabetic plus advanced glycation  $131.7 \pm 6.6$  DxA;  $P < 0.001$ ). There was also a reduction in staining observed in both control treatment groups as compared with the untreated control group ( $P < 0.05$ ).

Gene expression of the gp91phox subunit of NADPH oxidase was increased in diabetic animals as compared with controls (control  $1.1 \pm 0.3$ , diabetic  $2.8 \pm 0.5$  arbitrary units;  $P < 0.05$ ). This diabetes-induced expression was not attenuated by treatment with either ramipril or aminoguanidine (diabetic plus ACEI  $3.4 \pm 1.1$ , diabetic plus advanced glycation  $2.5 \pm 0.2$  arbitrary units). No differences among the control groups were demonstrated. **NF- $\kappa$ B.** The presence of activated NF- $\kappa$ B, as assessed by the EMSA in the renal cortex, was increased after 12 weeks of diabetes (control  $73.5 \pm 14.3$ , diabetic  $117.5 \pm 20.2\%$ ;  $P < 0.05$ ) (Fig. 7A and B). There was no attenuation of the activation of NF- $\kappa$ B in the diabetic kidney with either treatment (diabetic plus ACEI  $120.6 \pm 8.8$ , diabetic plus advanced glycation  $132 \pm 15.4\%$ ) (Fig. 7B).

## DISCUSSION

The present study has identified a relationship between the RAS and the accumulation of AGEs in experimental diabetic nephropathy. Diabetic animals treated with the ACEI ramipril for 12 weeks showed a reduction in renal fluorescence, serum fluorescence, and immunostaining for AGEs, similar to that observed in the diabetic group treated with the AGE formation inhibitor aminoguanidine. This attenuation of renal AGEs occurred in the context of reductions in both glomerular and tubular nitrotyrosine, a marker of protein oxidation. Gene expression of the gp91phox subunit of NADPH oxidase, activation of transcription factor NF- $\kappa$ B, and RAGE immunoreactivity were increased in the diabetic kidney but were not influenced by either treatment.

The classical view that angiotensin II primarily acts as a blood-borne hormone, exerting its effects via direct hemodynamic actions as a vasoconstrictor, has been increasingly disputed. Benefits of ACE inhibition beyond the hemodynamic effect have been noted by many authors (27,28), and recent human clinical trials have confirmed these benefits in man (29,30). In the present study, treatment with an ACEI during 12 weeks of experimental diabetes reduced albuminuria and systolic blood pressure, consistent with previous findings from us and other groups (31,32). Improvements in AER have also been previously described with an inhibitor of AGE formation, aminoguanidine, in experimental diabetic nephropathy (3). These two agents have conferred similar renoprotective effects, and it is postulated that the two agents may have similarities in their modes of action within the kidney, particularly in the context of diabetic nephropathy.

Blockade of the RAS by the AT1 receptor antagonist losartan has been shown previously to attenuate the accumulation of CML, a serum AGE, in a nondiabetic model of renal disease (13). Indeed, as previously demonstrated, renal impairment is closely associated with increases in serum AGE levels (33), presumably because the kidney plays a central role in AGE clearance. However, impaired renal function cannot be considered responsible in the present study because in this model, GFR is not

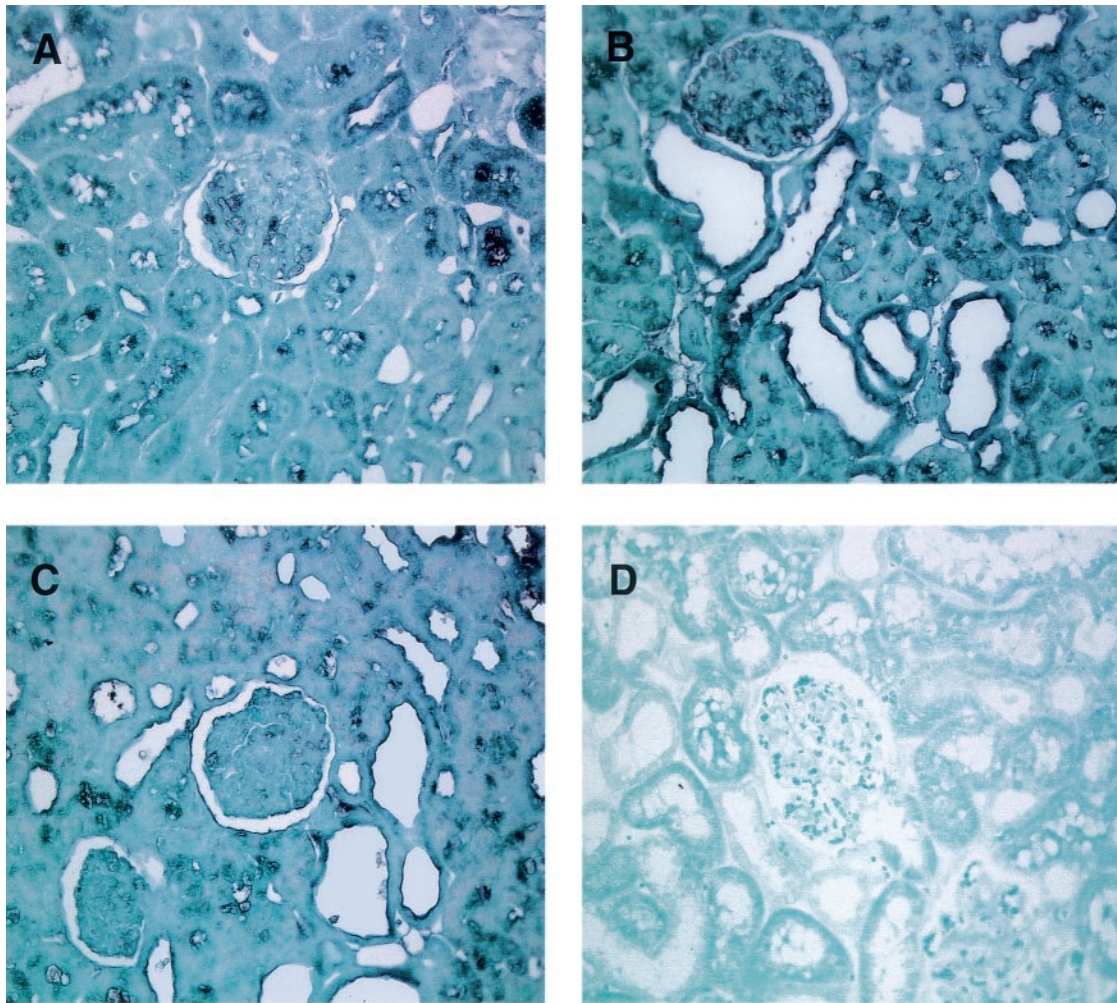


FIG. 6. Immunohistochemical staining for renal nitrotyrosine (A), control kidney (B), diabetic kidney (C), and diabetic kidney plus ACE inhibition (D). Magnification  $\times 400$ .

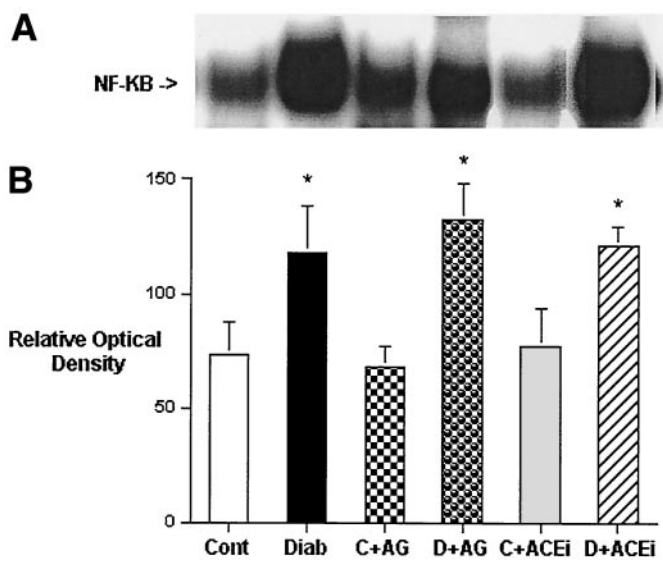


FIG. 7. A: Representative EMSA acrylamide gel pictured for all groups at 12 weeks. B: Quantitation of activated NF- $\kappa$ B by EMSA. \* $P < 0.05$  vs. control group. Cont, control; Diab, diabetic; C+AG, control plus aminoguanidine; D+AG, diabetic plus aminoguanidine; C+ACEi, control plus ACEi; D+ACEi, diabetic plus ACEi.

impaired in diabetes. Furthermore, the reduction in AGEs with ACE inhibition cannot be explained by increased GFR, since treatment with ramipril was associated with a modest decrease in GFR.

CML and pentosidine, a fluorescent AGE present in serum, are both produced by glycooxidation and require oxygen free radicals for their formation (4). Previously, it has been reported that increased oxidative stress is involved in the accumulation of AGEs in end-stage renal failure (34) and in diabetic glomerular lesions (10). More recently, Nishikawa et al. (5) have shown, in a series of in vitro experiments, that normalization of mitochondrial superoxide production blocks intracellular AGE accumulation and activation of NF- $\kappa$ B. Superoxide itself, however, is chemically inert (35) but, in combination with nitric oxide (NO), generates the highly reactive species, peroxynitrite ( $\text{OONO}^-$ ) (36). A characteristic reaction of  $\text{OONO}^-$  is the nitration of protein-bound tyrosine residues (35). In the present study, increases in glomerular and tubular nitrotyrosine were observed in our diabetic animals, which were attenuated by aminoguanidine. This may be due to aminoguanidine binding to Amadori compounds preventing the production of glucosone and consequently

the yielded by-product of this reaction, the superoxide anion (37). However, the finding that ramipril is equally effective in reducing the accumulation of nitrotyrosine suggests that another mechanism involving angiotensin II may also lead to blockade of free oxygen radical propagation. It is well established that aminoguanidine can also inhibit NO synthase, in particular the inducible isoform in diabetes, therefore decreasing NO production (38). Inhibition of endothelial NO synthase has recently been observed with the ACEI quinapril in experimental diabetic nephropathy (39). Therefore, less NO production could be an explanation for ramipril, and aminoguanidine reduced renal nitrotyrosine expression in the present study. It is unlikely that the changes observed with advanced glycation relate solely to effects on NO. First, agents that act to inhibit NO synthase such as L-NAME and methylguanidine without inhibiting AGE formation do not confer renoprotection (23). Second, agents that act as inhibitors of AGE formation without significantly inhibiting NO synthase isoforms have been shown to confer renoprotection in diabetes (19,40). Indeed, in another study, increases in NO synthase and consequent NO levels were reported after treatment with ramipril in nondiabetic Wistar Kyoto rats (41). Unfortunately, controversy remains with respect to the relationship between ACE inhibition and NO production.

Wolff et al. (42) have proposed that auto-oxidation of glucose may be a more likely candidate as the source of reactive oxygen in diabetes. Auto-oxidation produces glyoxal and arabinose from glucose along with superoxide radical and hydrogen peroxide. Glyoxal is a precursor for CML and arabinose a precursor of pentosidine, providing a logical route for the formation of AGEs and ROS via the Maillard reaction. In the present study, there was no amelioration of diabetes-induced increases in blood glucose or HbA<sub>1c</sub> with either treatment, indicating that the formation of AGEs was inhibited despite the absence of changes in plasma glucose concentrations. Alternative mechanisms to explain AGE-induced generation of ROS involve interactions of AGEs with RAGE, leading to the generation of ROS through stimulation of membrane-bound NADPH oxidase (8) and glycation of Cu,Zn superoxide dismutase (43). We have demonstrated increased gene expression of the membrane-bound gp91phox subunit of NADPH oxidase in this study, although no attenuation was seen with either treatment in the diabetic groups. This does not exclude a role for other NADPH oxidase subunits. Recently, Onazato et al. (39) have demonstrated increases in cytosolic p47phox after 2 weeks of streptozocin-induced diabetes, which was reduced by both quinapril and candesartan. It remains to be determined which specific oxidative pathways are activated in diabetes, as recent studies have suggested that a range of pathways are implicated in the increases in ROS in diabetes (44). For example, mitochondrial oxidation rather than generation of ROS via NADPH may be more important, as suggested by the studies of Nishikawa et al. (5), who emphasized the pivotal role of mitochondrial oxidation in diabetes complications. Indeed, deficiencies in mitochondrial respiratory complexes have been reported to increase superoxide radical generation (45). Specific *in vivo* testing of blockade of the production of mitochondrial free radical production and its link to glycoxidative processes

has not been performed. It still remains to be elucidated whether oxidative stress stimulates the production of AGEs or vice versa, or whether both processes are triggered simultaneously by an undefined stimulus.

Although RAGE protein expression was increased in diabetes, no attenuation of diabetes-induced increases in RAGE were found in diabetic treatment groups. The lack of effect on both gp91phox and RAGE are consistent with the findings of Wautier et al. (8), who demonstrated equivalent amounts of RAGE in both wild-type and gp91phox null mice exposed to AGEs. Gene expression of other AGE receptors, AGE R2 and AGE R3, remained unchanged in the various groups. This does not exclude changes in affinity because each of the ligands to these receptors have not been specifically identified to allow evaluation of the pharmacokinetics of the individual AGE receptor subtypes. Indeed, it remains to be determined whether these heterogeneous AGEs are the only endogenous ligands for the cloned AGE receptors, with a number of other ligands already identified for the receptor, RAGE (46).

Concomitant with increases in RAGE expression in the diabetic rats, there were increases in activation of the nuclear transcription factor NF- $\kappa$ B. Furthermore, no decrease in activated NF- $\kappa$ B was observed in the treated groups. An increase in RAGE expression in association with NF- $\kappa$ B activation is consistent with previous studies showing that AGEs activate NF- $\kappa$ B via RAGE. The evidence in this study, however, suggests that ramipril and aminoguanidine are not mediating their effects via blockade of RAGE- or NF- $\kappa$ B-dependent pathways. One possibility may be via blockade of the activation of protein kinase C (PKC), which has been suggested to play a pivotal role in the pathogenesis of diabetes complications (47). Our group has previously reported that aminoguanidine and ramipril prevent diabetes-induced increases in PKC activity in glomeruli, retina, and mesenteric artery (48), and it is therefore feasible to suggest that PKC may be involved.

In summary, the findings of the present study suggest an interaction between the RAS and advanced glycation in experimental diabetic nephropathy. In particular, there is evidence that ACE inhibition reduces the accumulation of renal and serum AGEs, possibly via effects on oxidative pathways. These findings support the concept that renoprotection afforded by both ACE inhibition and blockade of AGE formation involves common pathways such as inhibition of ROS formation/propagation.

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