

ALT-946 and Aminoguanidine, Inhibitors of Advanced Glycation, Improve Severe Nephropathy in the Diabetic Transgenic (mREN-2)27 Rat

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The severe diabetic nephropathy that develops in the hypertensive transgenic (mRen-2)27 rat with streptozotocin (STZ) diabetes has previously been considered angiotensin II-dependent. Because metabolic pathways are also activated in the diabetic kidney, the present study aimed to determine whether renoprotection could be afforded with inhibitors of advanced glycation end products (AGEs), ALT-946, and aminoguanidine (AG). At 6 weeks of age, nondiabetic control and STZ diabetic Ren-2 rats were randomized to receive vehicle, ALT-946 (1 g/l), or AG (1 g/l) and were studied for 12 weeks. Systolic blood pressure was unchanged with diabetes, ALT-946, or AG. Both kidney weight and glomerular filtration rate were increased with diabetes and unchanged with ALT-946 or AG. ALT-946 and AG equally ameliorated glomerulosclerosis and medullary pathology; however, ALT-946 did reduce cortical tubular degeneration to a greater extent than AG. Albumin excretion rate, which was elevated with diabetes, was reduced with ALT-946 but not AG. AGE immunolabeling was increased in glomeruli and reduced with ALT-946 and AG. These findings indicate that even in the context of renal injury presumed to be primarily blood pressure- and/or angiotensin II-dependent, approaches that interfere with metabolic pathways such as inhibitors of AGE formation can confer renal protection in experimental diabetes. *Diabetes* 51:3283–3289, 2002

D diabetic nephropathy involves progressive injury to both the glomerulus and the tubulointerstitium that is accompanied by increased protein excretion and ultimately a decline in renal function (1–3). It is likely that diabetic nephropathy occurs as a result of an interplay of hemodynamic and metabolic factors (4). However, it has not been ascertained whether

inhibitors of glucose-dependent pathways confer renal protection in experimental models of diabetes that seem to be highly dependent on hemodynamic factors such as the renin-angiotensin system (RAS) (5,6).

The RAS is implicated in the development of diabetic nephropathy, with the renoprotection afforded by RAS blockade being attributed to a reduction in systemic and intraglomerular pressure and suppression of the growth factor effects of a locally activated RAS (5–7). The hypertensive transgenic (mRen-2)27 rat that overexpresses the extrarenal RAS has been used by our group to study the pathogenesis of diabetic nephropathy. When made diabetic with streptozotocin (STZ), the Ren-2 rat develops a similar lesion to human diabetic nephropathy, including severe glomerulosclerosis, tubulointerstitial disease, and albuminuria (5,6,8,9). The diabetic renal lesion is associated with activation of both the glomerular and the proximal tubular RAS and overexpression of pro-sclerotic cytokines (5,6). Our finding that the injured kidney of the diabetic Ren-2 rat is improved but not completely protected with RAS blockade suggests that factors other than the RAS may participate in the progression of severe nephropathy in this diabetic model.

Advanced glycation end products (AGEs) are derived from the nonenzymatic glycosylation of long-lived proteins (10). This process is increased in diabetes and is viewed to contribute to the development of diabetes complications including nephropathy and retinopathy (11–14). Indeed, improvement of renal pathology is possible with agents such as aminoguanidine (AG), a hydrazine-like compound that blocks AGE formation by interacting with Amadori-derived products (11,14). A confounding factor when interpreting the beneficial effects of AG on diabetic renal pathology is that AG interacts with the nitric oxide (NO) pathway (15). AG has high structural homology with L-arginine, the substrate involved in the formation of NO, and AG has also been shown to inhibit NO synthase (NOS) (15,16). As increased NOS activity in the kidney may be involved in the renal hemodynamics of diabetes (17,18), the mechanisms responsible for the renoprotective effects described for AG are not readily apparent.

In vitro and in vivo studies have reported that ALT-946, N-(2-acetamidoethyl)hydrazinecarboximidamide hydrochloride is a more potent inhibitor of AGE-derived protein modification than AG (19). ALT-946 has advantages over AG as an inhibitor of AGE formation in that it has limited effects on NO production (19). With respect to diabetic

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AER, albumin excretion rate; AG, aminoguanidine; AGE, advanced glycation end product; GFR, glomerular filtration rate; GSI, glomerulosclerotic index; NO, nitric oxide; NOS, nitric oxide synthase; RAS, renin-angiotensin system; SBP, systolic blood pressure; STZ, streptozotocin.

nephropathy, a recent study by our group has shown that in normotensive diabetic rats with less advanced kidney disease, ALT-946 attenuated albuminuria in a manner similar to that observed with AG treatment (19). The objective of the present study was to evaluate the renoprotective effects of ALT-946 and AG in the diabetic transgenic Ren-2 rat that exhibits severe diabetic nephropathy in the setting of elevated blood pressure.

RESEARCH DESIGN AND METHODS

Six-week-old female heterozygous transgenic Ren-2 rats were randomized to be either diabetic or nondiabetic control. After an overnight fast, diabetes was induced by a single tail-vein injection of STZ (55 mg/kg diluted in 0.1 mol/l citrate buffer, pH 4.5; Sigma, St. Louis, MO), and nondiabetic control animals received 0.1 mol/l citrate buffer (5,6). Nondiabetic and diabetic Ren-2 rats were then randomized to receive no treatment (nondiabetic, $n = 8$; diabetic, $n = 7$), the inhibitor of AG formation, ALT-946 (1 g/l in drinking water; Alteon, Ramsay, NJ) (nondiabetic + ALT-946, $n = 8$; diabetic + ALT-946, $n = 8$), or AG hydrogen carbonate (1 g/l in drinking water; Fluka Chemica, Buchs, Switzerland) (nondiabetic + AG, $n = 7$; diabetic + AG, $n = 8$). These numbers of rats were used in the evaluation of all parameters. Treatments commenced from 2 days after STZ or control vehicle and continued for 12 weeks.

All experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes. All rats were allowed free access to tap water and standard rat diet (GR2, Clark-King & Co., Gladesville, NSW, Australia). Each week, rats were weighed and blood glucose (nondiabetic, 6 ± 1 mmol/l; diabetic, 27 ± 3 mmol/l, mean \pm SE) was estimated using an AMES glucometer (Elkhart, IN). Diabetic animals received a daily injection of insulin (4–6 units intraperitoneally; Ultratard, Novo Nordisk, Csestofte, Denmark) to promote weight gain and reduce mortality. Every 4 weeks, systolic blood pressure (SBP) was recorded in prewarmed conscious rats by tail-cuff plethysmography (20). Arterial pressure changes detected by a Pneumatic Pulse Transducer PE-300 (Narco Biosystems Inc., Houston, TX) were recorded using a Chart program (version 3.5) on a Maclab/2E System (AD Instruments, Pty Ltd., Castle Hill, Australia). SBP was taken at the same time of the day (1400–1700 h) to minimize circadian influences (5–6 h after treatment administration) from an average of at least three consecutive measurements to reduce variability (20).

Renal function. At 4, 8, and 12 weeks after STZ or control vehicle, Ren-2 rats were housed in metabolic cages (Iffa-Crede, L'Arbesle, France) for measuring 24-h fluid intake and urinary output as well as for collecting urine samples for subsequent estimations of albumin excretion rate (AER). During this time, rats continued to have free access to tap water and standard laboratory diet. AER was determined from an aliquot of urine by a double-antibody radioimmunoassay as previously described (5). Glomerular filtration rate (GFR) was determined by a single injection isotopic method (21) 1–2 days before the rats were killed. In brief, the isotope 99m technetium-diethylene triamine pentaacetic acid was injected into the tail vein and blood was sampled after 43 min. Plasma radioactivity was measured and compared with a reference prepared at the time of injection. GFR was calculated as clearance = $V \times \ln(P_o/P_t)/t$, where V is the volume of distribution, P_o is the plasma concentration at injection, and P_t is the observed plasma concentration at t minutes after injection (5).

Kidney histopathology. At 12 weeks after STZ or vehicle, Ren-2 rats were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg body wt intraperitoneally; Merial Australia Pty Ltd., Paramatta, Australia) and perfused via the abdominal aorta with 0.1 mol/l PBS (~150 ml [pH 7.4], 180–220 mmHg) for 1–2 min to remove circulating blood. The right kidney was then perfusion-fixed for 5 min with 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) (5). The kidney was then removed, sliced transversely, and postfixed overnight. After routine processing through graded alcohols, kidneys were embedded in paraffin and sectioned at 3 μ m. Sections stained with periodic acid Schiff reagent were used to evaluate basement membrane thickening, and Masson's trichrome was used for collagen deposition.

Glomerulosclerosis. For quantifying glomerulosclerosis, sections were stained with periodic acid Schiff reagent (5,6). A total of 250–300 glomeruli were randomly chosen from each rat kidney and carefully graded for sclerosis in a sequential manner so as not to grade the same glomerulus twice. Glomerulosclerosis was defined as glomerular basement membrane thickening, mesangial hypertrophy, and capillary occlusion. The degree of sclerosis in each glomerulus was graded subjectively on a scale of 0–4: grade 0, normal; grade 1, sclerotic area up to 25% (minimal); grade 2, sclerotic area 25–50% (moderate); grade 3, sclerotic area 50–75%; and grade 4, sclerotic area

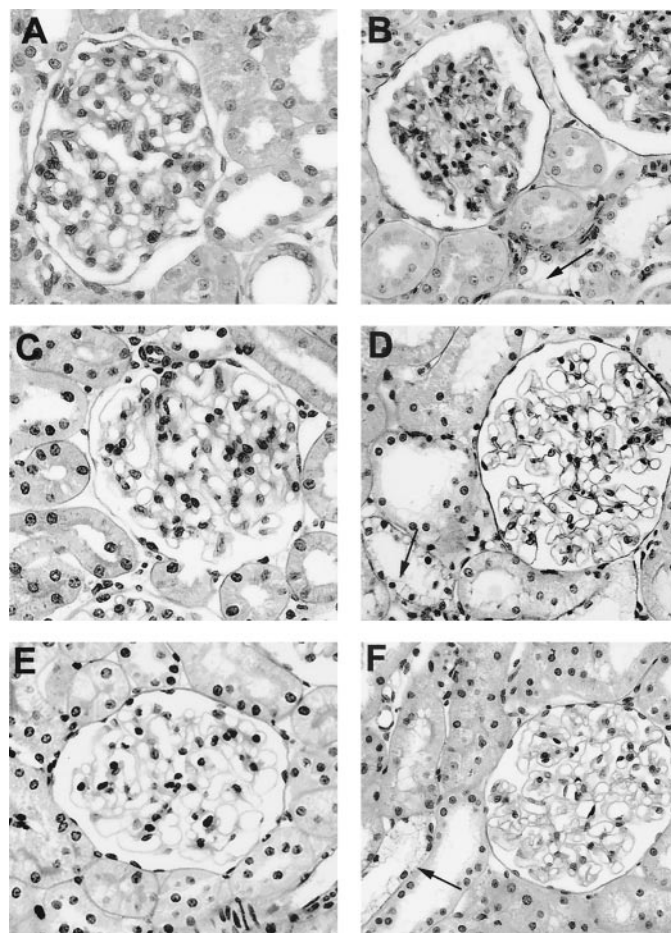


FIG. 1. Three-micron paraffin sections of kidney cortex from nondiabetic and STZ diabetic (mRen-2)27 rats treated with either ALT-946 or AG stained with periodic acid Schiff reagent. Magnification, $\times 350$. *A*: Untreated nondiabetic Ren-2 rat showing minimal glomerulosclerosis and normal tubules. *B*: Untreated diabetic Ren-2 rat exhibits glomerular damage and degenerated tubules (arrow). *C*: Nondiabetic Ren-2 rat treated with ALT-946. *D*: Diabetic Ren-2 rat treated with ALT-946 showing degenerated tubules (arrow) and minimal glomerular injury. *E*: Nondiabetic Ren-2 rat treated with AG. *F*: Diabetic Ren-2 rat treated with AG is similar to *D*.

75–100% (severe) (22). The glomerulosclerotic index (GSI) was then calculated using the following formula:

$$GSI = \sum_{i=0}^4 Fi(i)$$

where Fi is the percentage of glomeruli in the rat with a given score of (i).

Cortical tubular degeneration. Four paraffin sections were randomly chosen from one kidney from each animal and stained with Masson's trichrome. In each section, six fields of kidney cortex were randomly selected at a magnification of $\times 40$ using an Olympus BH-2 light microscope (Olympus, Tokyo, Japan). Degenerated tubules were identified in each field by the absence of cytoplasm (see Fig. 1). Results are expressed as the number of degenerated tubules per field of kidney.

Immunohistochemistry for AGEs. Three-micrometer paraffin sections of kidney were rehydrated and treated with 1% H_2O_2 /methanol followed by incubation in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, PA) for 20 min at room temperature. Sections were incubated with an anti-AGE antibody for 30 min at room temperature and then washed in PBS followed by incubation with 1:200 biotinylated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, CA). After another PBS wash and incubation with peroxidase-conjugated streptavidin (DAKO), peroxidase localization was revealed using diaminobenzidine tetrahydrochloride as the chromogen. The control for AGE labeling was lung from a 40-week-old rat. The AGE antibody (23) detects advanced glycated proteins including advanced glycated BSA and RNase. Studies to characterize the epitope of this antibody indicated that it detects

TABLE 1
Physical and biochemical parameters of nondiabetic and STZ diabetic (mRen-2)27 rats treated with either ALT-946 or AG

Ren-2 rats	<i>n</i>	Body weight (g)	Kidney weight (g)	SBP (mmHg)	GFR (ml/min)
Nondiabetic	8	319 ± 10.8	1.00 ± 0.05	188 ± 5	2.5 ± 0.2
Diabetic	7	230 ± 27*	1.37 ± 0.05*	187 ± 4	3.3 ± 0.2†
Nondiabetic + ALT-946	8	293 ± 5	0.98 ± 0.04	195 ± 6	2.3 ± 0.3
Diabetic + ALT-946	8	258 ± 6†‡	1.56 ± 0.05†	194 ± 8	3.4 ± 0.3†
Nondiabetic + AG	7	290 ± 5.6	0.98 ± 0.04	184 ± 5	2.1 ± 0.4
Diabetic + AG	8	205 ± 8*	1.49 ± 0.05†	203 ± 4	3.5 ± 0.4†

Values are expressed as mean ± SE. **P* < 0.01 compared with respective nondiabetic group; †*P* < 0.05 compared with respective nondiabetic group; ‡*P* < 0.01 compared with diabetic + AG.

carboxymethyllysine-containing proteins but does not detect native BSA, native RNase, or the AGE pentosidine (23).

Statistics. Comparisons of normally distributed variables between nondiabetic and diabetic groups were analyzed by one-way analysis of variance followed by a Fisher's individual post hoc comparison. All statistics were performed using Minitab version 10.5. A change was considered statistically significant at *P* < 0.05. All values are expressed as mean ± SE, except for albuminuria, which are shown as geometric means $\bar{x} \div$ tolerance factors. The minimum number of animals required for statistical significance is six rats per group that was estimated using an α value of 0.05 and a β value of 0.1.

RESULTS

Body weight, kidney weight, and SBP. The results are summarized in Table 1. In untreated diabetic Ren-2 rats, body weight was reduced compared with nondiabetic controls. In diabetic Ren-2 rats, neither ALT-946 nor AG restored body weight to nondiabetic control. Kidney weight was increased with diabetes and unchanged with either ALT-946 or AG. SBP was similar in nondiabetic and diabetic Ren-2 rats and unchanged with either ALT-946 or AG.

Kidney function. GFR was increased with diabetes and unchanged with ALT-946 or AG (Table 1). At 12 weeks of diabetes, AER was increased by 760% compared with untreated and treated nondiabetic Ren-2 rats (Fig. 2). In diabetic Ren-2 rats, AG treatment did not improve AER. However, ALT-946 attenuated the increase in AER by 250%, but this was still higher than observed in nondiabetic Ren-2 rats. Neither treatment influenced AER in the nondiabetic animals.

Kidney histopathology. There were no significant ultrastructural abnormalities in kidneys from untreated nondiabetic Ren-2 rats, except for occasional thickened basement membranes (Figs. 1A and 3A). In untreated diabetic Ren-2 rats, many glomeruli exhibited thickened

basement membranes, occluded capillaries, and mesangial expansion, and this was reflected by an increased GSI (Figs. 1B and 3A). In the cortical interstitium of some kidneys from untreated diabetic Ren-2 rats, collagen deposition and inflammatory cells were increased compared with nondiabetic controls (not shown). ALT-946 and AG treatment of nondiabetic Ren-2 rats did not alter tissue morphology, and GSI was similar to untreated nondiabetic controls (Figs. 1C and E and 3A). In kidneys from diabetic Ren-2 rats treated with either ALT-946 (Fig. 1D) or AG (Fig. 1F), fewer glomeruli were damaged compared with untreated diabetic Ren-2 rats, and GSI in both groups was reduced to the level of nondiabetic controls (Fig. 3). The cortical interstitium from diabetic Ren-2 rats treated with either ALT-946 or AG seemed similar to untreated nondiabetic rats. The kidney medulla of untreated diabetic Ren-2 rats contained increased tubulointerstitial collagen staining (Fig. 4). ALT-946 and AG improved medullary pathology to a similar extent in diabetic Ren-2 rats (Fig. 4)

Kidney cortical tubular degeneration. In nondiabetic Ren-2 rats that were either untreated or treated with ALT-946 or AG, degenerated tubules were not observed in the kidney cortex (Figs. 1A, C, and E and 3B). However, in untreated diabetic Ren-2 rats, numerous degenerated tubules were observed (Figs. 1B and 3B), and these were decreased with AG (Figs. 1F and 3B) and further reduced with ALT-946 (Figs. 1D and 3B).

AGE immunohistochemistry. In untreated nondiabetic Ren-2 rats, AGE was localized to glomeruli and some cortical tubules (Fig. 5A). With diabetes, AGE labeling was intensified in glomeruli (Fig. 5B) and was evident in distal tubules and interstitium. ALT-946 and AG treatment reduced AGE labeling in glomeruli and tubulointerstitium of diabetic Ren-2 rats (Fig. 5C and D).

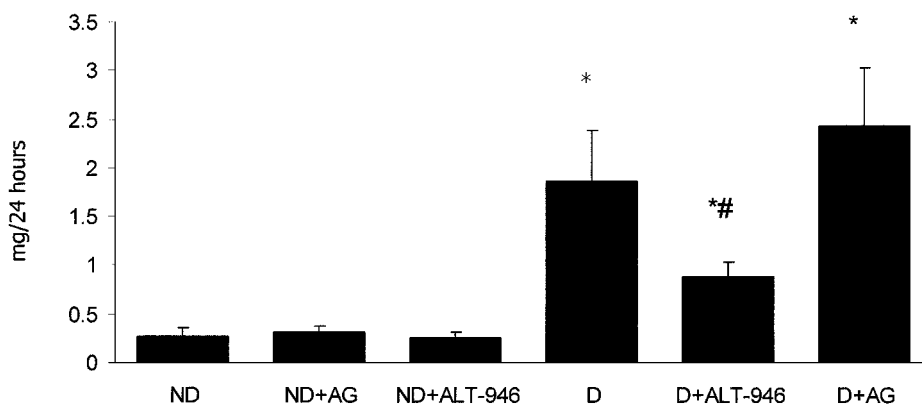


FIG. 2. 24-h AER at 12 weeks after induction of control or STZ diabetes in female heterozygous transgenic (mRen-2)27 rats treated with either ALT-946 or AG. Values are geometric means $\bar{x} \div$ tolerance factors; *n* = 7–8 rats/group. ND, nondiabetic; D, diabetic. **P* < 0.01 compared with all ND groups; #*P* < 0.01 compared with D and D+AG.

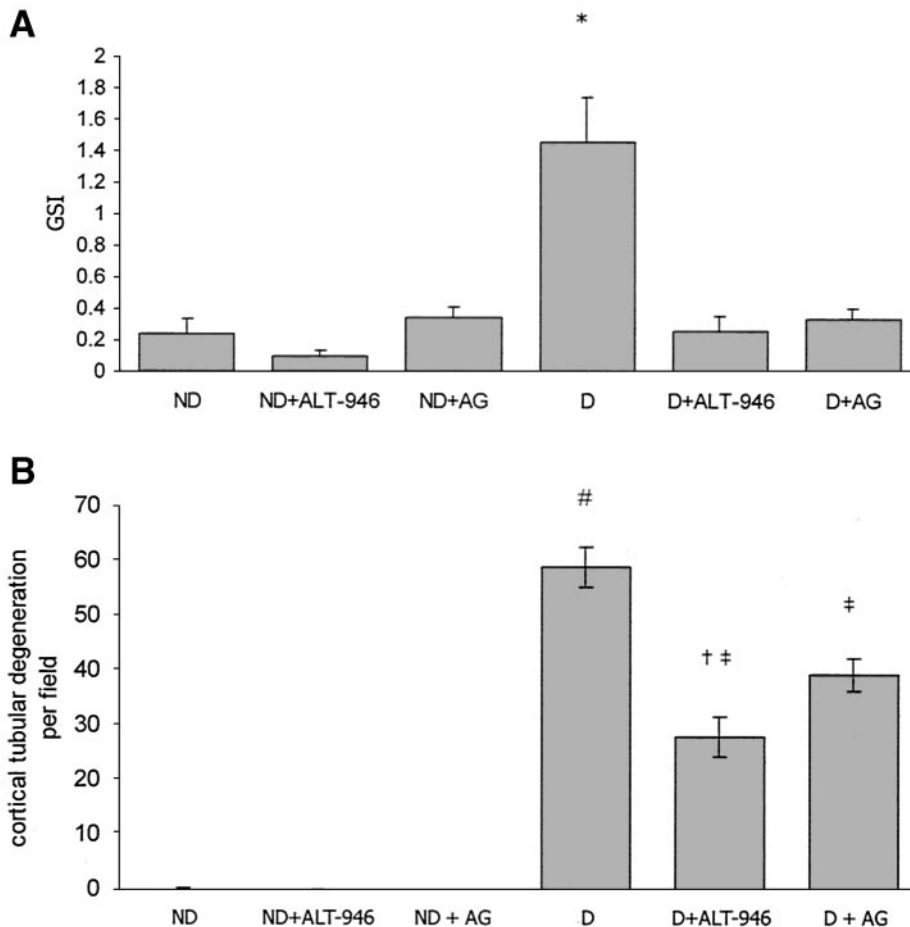


FIG. 3. GSI (A) and cortical tubular degeneration (B) in kidneys from nondiabetic and STZ diabetic (mRen-2)27 rats treated with either ALT-946 or AG. Values are mean \pm SE; $n = 7-8$ rats/group. ND, nondiabetic; D, diabetic. * $P < 0.01$ compared with all groups; # $P < 0.0001$ compared with all ND groups and D; † $P < 0.05$ compared with D+AG.

DISCUSSION

The present study has shown that inhibitors of AGE formation such as ALT-946 and AG reduce severe glomerulosclerosis and cortical tubular degeneration. This occurred in the context of reduced renal AGE accumulation without influencing the elevated blood pressure characteristic of the diabetic transgenic Ren-2 rat. Furthermore, the ALT-946 provided additional renoprotection by ameliorating albuminuria in diabetic animals. These findings suggest that even in the context of systemic hypertension, activation of the local RAS, and chronic hyperglycemia, it is possible to attenuate renal injury by inhibiting AGE formation. These data further emphasize the pivotal role that advanced glycation plays in the pathogenesis of diabetic nephropathy.

The pathogenetic effects of renal AGE accumulation in diabetes have been shown in a number of studies (10,24,25). AG has been reported to attenuate mesangial expansion and albuminuria in animal models of diabetic renal disease (11,14,26). Other AGE inhibitors such as OPB 9195 and 2,3 diaminophenazine prevent renal structural injury and mesenteric vascular hypertrophy, respectively (27-29). Recently, a new class of AGE inhibitors that are more effective in blocking AGE accumulation in tissues was developed (19). ALT-946 belongs to this group of compounds, and we have previously shown it to be a potent inhibitor of renal AGE accumulation and to reduce glomerulosclerosis and albuminuria in a normotensive diabetic model with less advanced renal injury (19). In

agreement with that study, both ALT-946 and AG markedly reduced AGE accumulation in glomeruli of the diabetic Ren-2 rat. Of particular interest was the observation that both compounds, despite not reducing the hypertension of the diabetic Ren-2 rat, improved severe glomerulosclerosis, as has been reported using agents that interrupt the RAS (5,6,9). This supports the concept that systemic and intraglomerular pressure are not entirely responsible for the development of severe glomerulosclerosis in the diabetic Ren-2 rat. Indeed, we have previously shown that combined blockade of the endothelin type A and endothelin type B receptors ameliorates hypertension in the diabetic Ren-2 rat but not glomerulosclerosis (6).

The major difference in the renoprotective effects between ALT-946 and AG in the diabetic Ren-2 rat is their effect on AER. Unlike ALT-946, AG did not improve albuminuria in diabetic Ren-2 animals. The reasons for this are unclear; however, AG has been reported to have variable effects on albuminuria that may be time-dependent (14,30). For instance, AG prevents albuminuria in STZ diabetic Sprague Dawley rats at 32 weeks but not earlier at 8 and 16 weeks (14). Therefore, it is possible that the 12-week experimental period used in the present study may not have been long enough to reveal the anti-albuminuric effects of AG. However, one cannot exclude that ALT-946 is a more effective inhibitor of AGEs than AG. We have previously shown ALT-946 to be more potent than AG in reducing AGE-protein cross-linking both in vitro and in vivo (19).

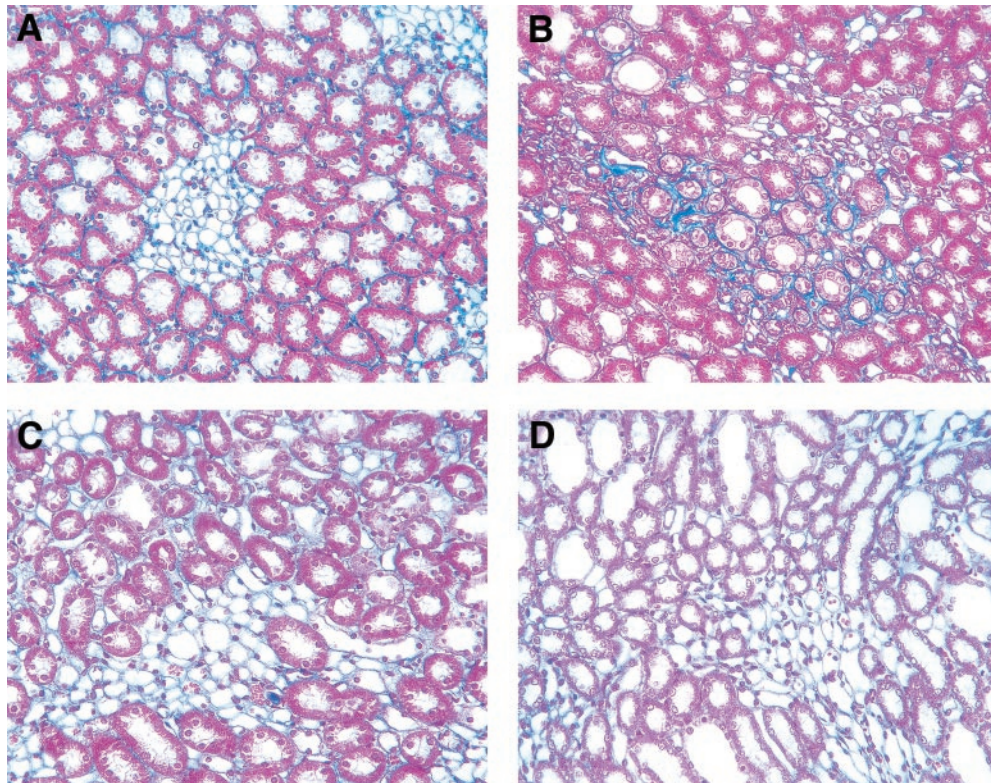


FIG. 4. Three-micron paraffin sections of kidney medulla from nondiabetic and STZ diabetic (mRen-2)27 rats treated with either ALT-946 or AG. Stained with Masson's trichrome. Magnification, $\times 350$. **A:** Untreated nondiabetic Ren-2 showing minimal collagen deposition (blue). **B:** Untreated diabetic Ren-2 showing increased collagen deposition in the tubulointerstitium. **C:** Nondiabetic Ren-2 rat treated with ALT-946 is similar to **A**. **D:** Diabetic Ren-2 rat treated with AG is similar to **A** and **C**.

Another possibility for the renal superiority of ALT-946 over AG is that this agent has a minimal effect, in contrast to AG, on various NOS isoforms. Because NO is a potent renal vasodilator, it is possible, though not proved, that AG's action as an NOS inhibitor could counteract some of the beneficial effects of this agent as an inhibitor of AGE formation. This—together with our previous findings that ALT-946 reduces renal AGE accumulation and albuminuria in the diabetic Sprague Dawley rat—indicates that the beneficial effects of ALT-946 and AG on kidney pathology occur independent of NO, primarily by inhibiting AGE accumulation (19). This concept is consistent with findings in diabetic Sprague Dawley rats, in which inhibitors of NOS, *N*-nitro-*L*-arginine methyl ester (*L*-NAME), and methylguanidine were not as effective as AG at conferring renoprotection (23).

A novel feature of the diabetic renal lesion that develops in the transgenic Ren-2 rat is extensive cortical tubular degeneration, which we have recently shown to represent apoptosis of proximal tubule cells (5,31). In the present study, both ALT-946 and AG reduced cortical tubular degeneration with the largest benefit observed with ALT-946. The importance of AGEs in mediating tubulointerstitial injury was suggested recently by our group in a series of *in vitro* and *in vivo* studies (32). *In vitro*, prepared AGEs, including carboxymethyl lysine–modified derived proteins, were shown in a dose- and time-dependent manner to induce proximal tubule transdifferentiation (32). This phenomenon was also observed *in vivo* and could be attenuated by another approach that reduces renal AGE accumulation using a cross-link breaker (32). AGEs may

also influence apoptosis of renal tubular cells as has been shown in other cell types, such as pancreatic β -cells and human umbilical vein endothelial cells (33,34). Although effective, ALT-946 and AG did not improve the tubular lesion in the diabetic Ren-2 rat to the same extent as RAS blockade (5,9). The proximal tubule has been shown by us and other investigators to contain a local RAS that is upregulated in chronic hyperglycemia (5,6,35,36). In the diabetic Ren-2 rat, it is likely that angiotensin II is a major contributor to the apoptosis of proximal tubular cells either directly or via other injurious cytokines, such as transforming growth factor- β and platelet-derived growth factor (4,6). Taken together, these findings suggest that in the diabetic Ren-2 rat, the development of cortical tubular degeneration is influenced by AGEs, but the main pathogenetic factor is likely to be angiotensin. Angiotensin II remains a major factor promoting tubulointerstitial injury in various pathological states, including diabetes (37,38).

In conclusion, we provide evidence that inhibitors of AGEs such as ALT-946 and AG improve severe diabetic kidney disease in a model that had previously been shown to be angiotensin II–dependent. This study in the hypertensive diabetic Ren-2 rat shows that these compounds exert their beneficial effects on kidney structure and function by a mechanism independent of blood pressure reduction. The ability of agents that inhibit AGE formation to attenuate a form of renal injury that is considered to be angiotensin II–dependent provides additional evidence that diabetic nephropathy occurs as a result of an interplay of metabolic and hemodynamic factors (4). Further elucidation of this interaction will greatly assist in defining

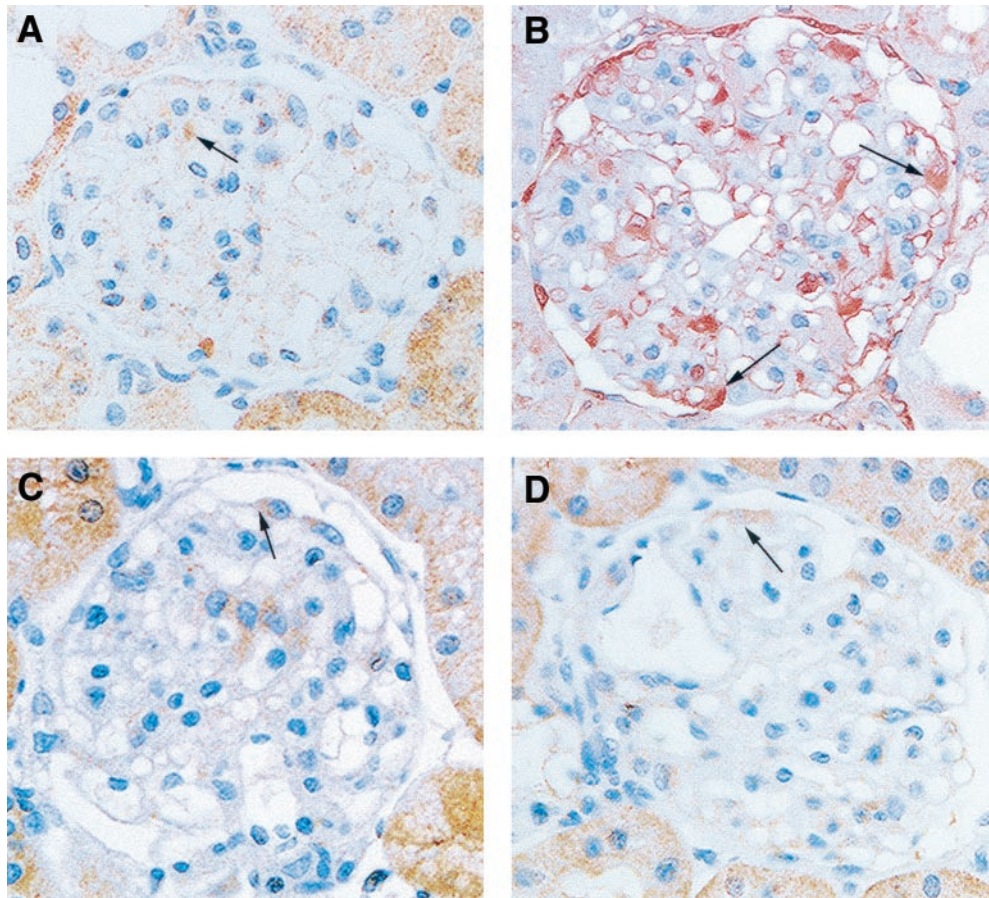


FIG. 5. Three-micron paraffin sections of kidney immunolabeled with AGE from nondiabetic and STZ diabetic (mRen-2)27 rats. Counterstain, hematoxylin. Magnification, $\times 420$. **A:** Untreated nondiabetic Ren-2 rat. **B:** Untreated diabetic Ren-2 rat showing increased AGE immunolabeling in the glomerulus (arrows). **C:** Diabetic Ren-2 rat treated with ALT-946. **D:** Diabetic Ren-2 rat treated with AG showing minimal AGE immunolabeling in the glomerulus (arrow).

critical downstream events that mediate angiotensin II- and AGE-induced renal lesions in diabetes.

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