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Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats

(renal insufficiency/diabetes/aging/animal model/aminoguanidine)

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ABSTRACT **High levels of tissue advanced glycation end products (AGEs) that result from the spontaneous modification of proteins by glucose occur in diabetes and aging. To address the potential pathogenic role of AGEs in the glomerulosclerosis of diabetes or nephrosclerosis of aging, doses of AGE-modified rat albumin (25 mg per kg per day, i.v.) sufficient to elevate circulating AGE levels to the range of diabetic serum were administered daily to healthy rats alone or in combination with the AGE inhibitor aminoguanidine. After 5 months, the AGE content of renal tissues in AGE-treated rats rose to 50% above controls ($P < 0.025$), whereas serum contained 2.8-fold greater AGE levels ($P < 0.025$). Light and electron microscopy of kidneys from AGE-treated rats revealed a more than 50% increase in glomerular volume compared to controls ($P < 0.001$), significant periodic acid/Schiff reagent-positive deposits, basement membrane widening, and mesangial extracellular matrix increase and indicated significant glomerulosclerosis compared to untreated ($P < 0.002$) or albumin-treated controls ($P < 0.002$). These changes were associated with significant loss of protein ($P < 0.005$) and albumin ($P < 0.002$) in the urine of AGE-treated rats compared to controls. Cotreatment with aminoguanidine markedly limited both the structural and functional defects. These *in vivo* data demonstrate that AGEs influence glomerular structure and function in a manner leading to glomerulosclerosis. The effects are AGE-specific, as they are ameliorated by a pharmacological AGE inhibitor, aminoguanidine.**

A subgroup of patients with diabetes mellitus may experience glomerulosclerosis as a result of mesangial expansion, a process that can progress to renal failure (1). The proximal cause of this process differs from that of the nephrosclerosis of aging, which more likely reflects the effects of vascular lesions with atrophic glomeruli and ischemia (2). Recent clinical evidence indicates that reduction of mean blood glucose level can significantly lower the risk for development of certain diabetic complications including renal disease (3). This confirmed that sustained hyperglycemia, reflected in an increased rate of nonezymatic glycation of proteins, is a prerequisite for the development of long-term diabetic complications. Advanced glycation end products (AGEs), the late products of the covalent modification of proteins by glucose, have also been shown to accumulate slowly in renal and extrarenal tissues as a function of age and at a more rapid rate in diabetes mellitus (4–6). Numerous *in vitro* studies have suggested that by direct chemical modification and crosslinking of matrix and plasma proteins or through interactions with cell surface AGE-specific receptors, AGEs may be capable of inducing tissue damage leading to diabetic complications (6, 7).

Short-term *in vivo* animal studies have in part confirmed this pathogenic link by showing that the administration of exogenous AGEs to normal rats and rabbits for a brief period (2–4 weeks) results in systemic vasculopathy (8), while in normal SJL mice AGEs enhance the expression of a1IV collagen and laminin B1 mRNAs within the glomerulus (9). However, the long-term impact of cumulative AGE deposition on the structure and function of the kidney has remained obscure.

In this report, we present evidence indicating that prolonged *in vivo* exposure of normal renal tissues to AGE-modified homologous serum albumin induces marked renal lesions and that certain aspects of this pathology are inhibited by the coadministration of aminoguanidine.

METHODS

Preparation of Advanced Glycation Products. Rat serum albumin (RSA) (Sigma) was passed over an Affi-Gel Blue column (Bio-Rad), a heparin-Sepharose CL-6B column (Pharmacia), and an endotoxin-binding affinity column (Detoxigel, Pierce) to remove possible contaminants (8, 9). RSA modified by AGEs was prepared as described (8, 9). AGE levels were measured by an AGE-specific ELISA (10) (AGE-rat albumin, 62 AGE units/mg of protein; unmodified rat albumin, 1.2 units/mg). Each reagent contained endotoxin (E-Toxate, Sigma) at <0.2 ng/ml.

Animal Studies. Male Sprague–Dawley rats (150 g) aged 3 months ($n = 50$) (Charles River Breeding Laboratories) were used in these studies, which were conducted in accordance with The Picower Institute Laboratory Animal Center guidelines. After a 2-week adaptation period, rats were given tail vein injections with AGE-modified or native RSA (25 mg per kg per day) or with AGE-RSA followed by infusions of aminoguanidine hydrochloride (100 mg per kg per day, i.v.) for 5 months. Serum samples were collected at the end of the treatment period from all groups for serum AGE determination by an AGE-specific ELISA (10). To determine the amount of AGE that accumulated within the kidneys, animals ($n = 5$ per group) were sacrificed by exposure to CO_2 and their kidneys were removed rapidly, weighed, and sectioned. Approximately one-quarter of each kidney was finely minced, delipidated with acetone/chloroform, 1:1 (vol/vol), and digested with 1% type VII collagenase (Sigma) for 48 h at 37°C for determination of collagen content as described (11). Tissue AGE levels were determined by ELISA (10).

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Abbreviations: AGE, advanced glycation end product; RSA, rat serum albumin; GBM, glomerular basement membrane; PAS, periodic acid/Schiff reagent; TGF-β1, transforming growth factor β1.

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Twenty-four-hour urine samples were obtained for three consecutive days before sacrifice for total creatinine, total protein (Bio-Rad), and total albumin determinations by an indirect ELISA using rabbit anti-rat albumin (Cappel) as primary antibody (1:1000 dilution) and goat anti-rabbit IgG (1:3000 dilution) (Boehringer Mannheim) as secondary antibody. Creatinine measurements were performed by a quantitative colorimetric assay (Stanbio Laboratory, San Antonio, TX).

Light Microscopy. After perfusion with saline, kidney sections (3-mm wedge) were fixed in ice-cold 10% (vol/vol) buffered formalin (Millonig's modified buffered formalin, pH 7.4) for 24 h. Paraffin-embedded tissue sections from all animal groups (n = 5 per group, including a 3-month- and an 8-month-old group of nontreated controls) were processed in triplicate by staining with Harris' alum hematoxylin, periodic acid/Schiff reagent (PAS), and Yajima stains for routine histological examination in a blinded fashion. The sections were scored by two investigators for mesangial expansion, global and segmental glomerulosclerosis, Bowman's capsule thickening, tubular, interstitial, and arteriolar changes. Values used to relate the apparent increase in mesangial volume were as follows: 1+ for a 2-fold increase, 2+ for a 3-fold increase, etc. Glomerular profiles (mean of 94 per animal) were evaluated for segmental and global glomerulosclerosis. Glomerular volumes were calculated as described (12).

Electron Microscopy. Tissues were fixed in ice-cold 2.0% (vol/vol) glutaraldehyde (0.1 M sodium cacodylate, pH 7.3), postfixed for 1 h in buffered osmium tetroxide (1.0%), prestained in 0.5% uranyl acetate for 1 h, and embedded in Effapoxy resin (E. F. Fullam, Latham, NY). Ultrathin sections were examined by a JEOL 100CXII electron microscope.

Statistical Analyses. In general, comparisons between groups were carried out by a two-way analysis of variance (ANOVA). Comparisons from measurements not normally distributed (mesangial volume and glomerulosclerosis) were completed by the Mann–Whitney U test and are expressed as the mean ± SEM.

RESULTS

During the entire study period all animals gained weight normally. The daily treatment of rats with AGE-RSA over a period of 5 months resulted in renal-tissue AGE accumulation (≈50%), compared to kidneys from RSA-treated ($P < 0.05$) or untreated control ($P < 0.025$) rats (Fig. 1*A*). In animals cotreated with the AGE-crosslinking inhibitor aminoguanidine, collagen-associated AGE levels did not increase above normal (P = not significant). Circulating AGE-protein levels in AGE-RSA-treated rats were 2.8-fold higher than in untreated controls ($P < 0.05$), whereas in the AGE-RSA/Ag-treated group, serum AGE values fell between the AGE-RSA and control group values (Fig. 1*B*). A 2.5-fold increase in total urinary protein excretion was associated with AGE-RSA treatment, compared to the control ($P < 0.001$) and unmodified-RSA-treated groups ($P < 0.01$). The addition of aminoguanidine was associated with a significant, although not complete inhibition of this proteinuria ($P < 0.025$ vs. AGE-RSA) (Fig. 1*C*). Similarly, urinary albumin excretion in the AGE-RSA-treated rats was 2-fold greater than in the RSA-treated ($P < 0.005$) or the untreated control ($P < 0.001$) rats; in contrast, AGE-RSA/aminoguanidine rats had near normal urinary albumin levels (Fig. 1*D*). Mean urinary creatinine clearance values among the groups were not significantly different (data not shown). However, urinary excretion of AGE-immunoreactive substances was significantly higher in groups treated with AGE-RSA and AGE-RSA/aminoguanidine, compared to untreated control ($P < 0.001$ and < 0.005, respectively) and RSA-treated ($P < 0.05$ and < 0.025, respectively) rats.

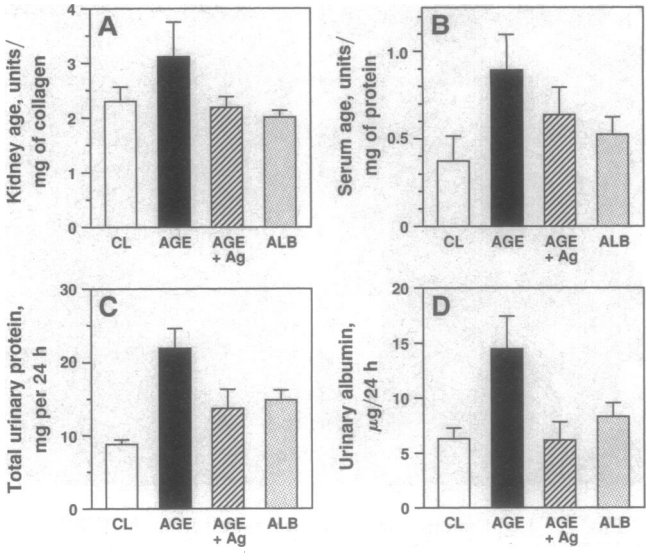

FIG. 1. Rat kidney tissue (*A*) and serum (*B*) AGE levels and total urinary protein (*C*) and albumin excretion (*D*) after treatment with AGE-modified RSA (AGE), unmodified RSA (ALB), or AGE-RSA/aminoguanidine (AGE/Ag) for 5 months. CL, control. (*A*) Kidney AGE levels (mean ± SEM, n = 6–12 per group). Significant P values: AGE vs. CL, <0.05; AGE vs. ALB, <0.025; AGE vs. AGE/Ag, <0.025. (*B*) Serum AGE levels (mean ± SEM). P values: AGE vs. CL, <0.05; AGE vs. ALB, <0.025. Total urinary protein (*C*) and albumin (*D*) concentrations are expressed as the mean ± SEM. (*C*) P values: AGE vs. AGE/Ag, <0.025; AGE vs. ALB, <0.01; AGE vs. CL, <0.001; AGE/Ag vs. CL, <0.05. (*D*) P values: AGE vs. AGE/Ag, <0.005; AGE vs. ALB, <0.01; AGE vs. CL, <0.001.

Silver- and PAS-stained sections from rats treated with AGE-RSA revealed the presence of some obsolescent glomeruli, while others contained areas of sclerosis with synechiae. Many large PAS-positive deposits were found in these segmental lesions (Fig. 2*A*), yielding a significant proportion of severely affected glomeruli in this group, compared to nontreated controls ($P < 0.002$) or to those animals injected with RSA alone ($P < 0.005$) (Table 1). Some mesangial regions contained an increased amount of mesangial matrix without significant hypercellularity. Bowman's capsules and tubular basement membranes were often thickened by PAS-positive material (Fig. 2*A*). There were occasional cellular infiltrates, mainly around the vessels.

In the AGE-RSA/aminoguanidine-treated group only a few glomeruli had similar lesions ($P < 0.002$ vs. AGE-RSA) (Table 1 and Fig. 2*D*). The tendency for mesangial expansion, however, was not influenced by aminoguanidine cotreatment (Table 1). None of the nontreated or RSA-treated animals showed any lesions (Fig. 2 *B* and *C*).

AGE-RSA administration was associated with a marked (>50%) increase in glomerular volume above the age-matched 8-month-old ($P < 0.001$), the 3-month-old ($P < 0.001$) control, and the RSA-treated control ($P < 0.001$) groups (Fig. 3). These glomerular volume changes were prevented to a significant extent (by 75%) in rats given AGE-RSA/aminoguanidine ($P < 0.001$).

Electron microscopic evaluation of selected tissue sections showed substantial glomerular basement membrane (GBM) widening and mesangial matrix enlargement in some glomeruli of kidneys exposed to AGE-RSA (Fig. 4 *A* and *B*), compared to the minimal changes seen in the age-matched untreated animals (Fig. 4*C*). AGE-associated loss of the normal three-layered structure of GBM was frequently accompanied by large electron-dense deposits distributed pri-

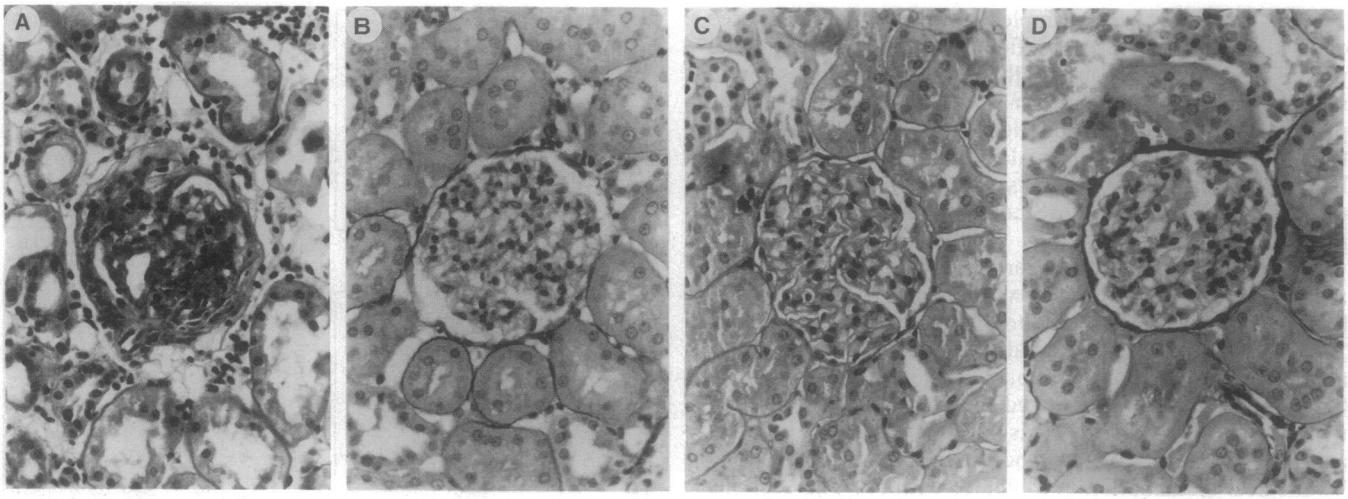

FIG. 2. Light microscopy of rat glomeruli from AGE-RSA-treated (*A*), untreated control (*B*), unmodified-RSA-treated control (*C*), and AGE-RSA/aminoguanidine-treated (*D*) rats. (PAS stain; ×75.)

marily in the mesangium and extending into the subendothelial area (Fig. 4*A*). In many instances, the matrix increase resulted in partial occlusion of glomerular capillaries (Fig. 4*B*) and was associated with podocyte spreading (Fig. 4*A*). These alterations, although not completely abrogated, were noticeably less prominent in sections of kidneys exposed to the simultaneous infusion of AGE-RSA/aminoguanidine (Fig. 4*D*).

DISCUSSION

The present studies demonstrate that chronic administration of *in vitro*-prepared protein-AGEs to otherwise healthy rats leads to advanced pathological changes in renal glomerular structure and function consistent with focal glomerulosclerosis and albuminuria. Beyond the existing evidence implicating AGEs in a number of biochemical, cellular, and pathophysiological abnormalities (7), these data offer confirmatory evidence for a cause and effect relationship between long-term AGE accumulation and renal pathology.

Given that diabetes is a pathogenically complex disease, the specific *in vivo* role of late glycation products (AGEs) in the establishment of advanced glomerular lesions has not been fully determined. A much less well characterized entity is the chronic nephrosclerosis associated with normal aging (2). A general agreement exists regarding the association of progressive albuminuria with increasing GBM permeability and thickening, the development of vascular lesions, and the eventual emergence of focal and global glomerular sclerosis (2, 13). The potential contribution of an age-dependent accumulation of AGEs to the aging kidney dysfunction and structural damage is even less well understood.

Table 1. Individual glomerular profiles of AGE-treated rat kidneys

Group	Focal segmental glomerular sclerosis, %	Mesangial volume, %
AGE-RSA	9.0 ± 11.2	0.9 ± 0.6
RSA	0.5 ± 1.1	0.6 ± 0.5
AGE-RSA/Ag	0.7 ± 0.8	0.9 ± 0.7
Control	0.0	0.0

Ag, aminoguanidine. *P* values for focal segmental glomerular sclerosis are as follows: 0.0021, AGE-RSA vs. RSA; 0.0017, AGE-RSA vs. AGE-RSA/Ag; 0.002, AGE-RSA vs. control; 0.08, AGE-RSA/Ag vs. control. *P* values for mesangial volume are as follows: 0.39, AGE-RSA vs. RSA; 0.89, AGE-RSA vs. AGE-RSA/Ag; 0.43, AGE-RSA vs. control; 0.62, AGE-RSA/Ag vs. control.

Earlier studies began to explore these issues by injecting superphysiologic amounts (100 mg per kg per day) of species-specific AGE-modified serum albumin to normal rats and rabbits over a short period (4–8 weeks) (8). This regimen resulted in widespread vascular leakage and vasodilatory impairment. Subsequent 3- to 4-week-long studies in mice aimed at detecting an early pattern of AGE-induced gene dysregulation demonstrated a significant increase in selected components of extracellular matrix (α1IV collagen and laminin B1) and tissue growth factor (transforming growth factor β1) mRNA expression, in association with glomerular hypertrophy (9). These early changes in renal cell growth responses pointed to a process by which more extended exposure to AGEs could eventually result in marked extracellular matrix accumulation.

To study the long-term effects of AGE, the daily AGE administration was adjusted to a lower dose (25 mg per kg per day), sufficient to maintain circulating AGE levels close to the 2-fold above normal range known to occur in human diabetes with reasonably intact renal function (10, 14). This permitted the observation of long-term effects of modestly elevated levels of AGEs on renal structure and function, as might occur in diabetes, but in the absence of hyperglycemia. Although intact exogenous AGE-RSA is certain to account in part for the circulating AGEs, a significant portion of it is likely to reflect degradation products. This processed material is eventually cleared via the kidney, as AGE-rich peptide fragments (14). This was demonstrated by a marked increase (4-fold above control) in the AGE-immunoreactive material

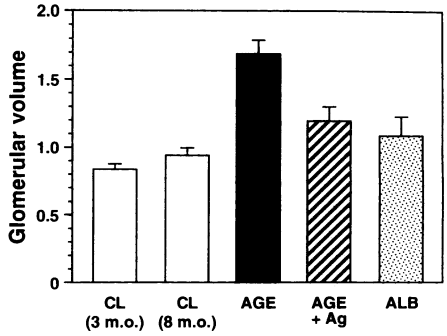

FIG. 3. Glomerular hypertrophy induced by AGE-RSA. Volume data ($mm^3 \times 10^6$) are the mean ± SEM of each group. Significant *P* values: AGE vs. AGE/aminoguanidine (AGE/Ag), ALB, or either control (CL), <0.001; AGE/Ag vs. controls, <0.05. m.o., Months old.

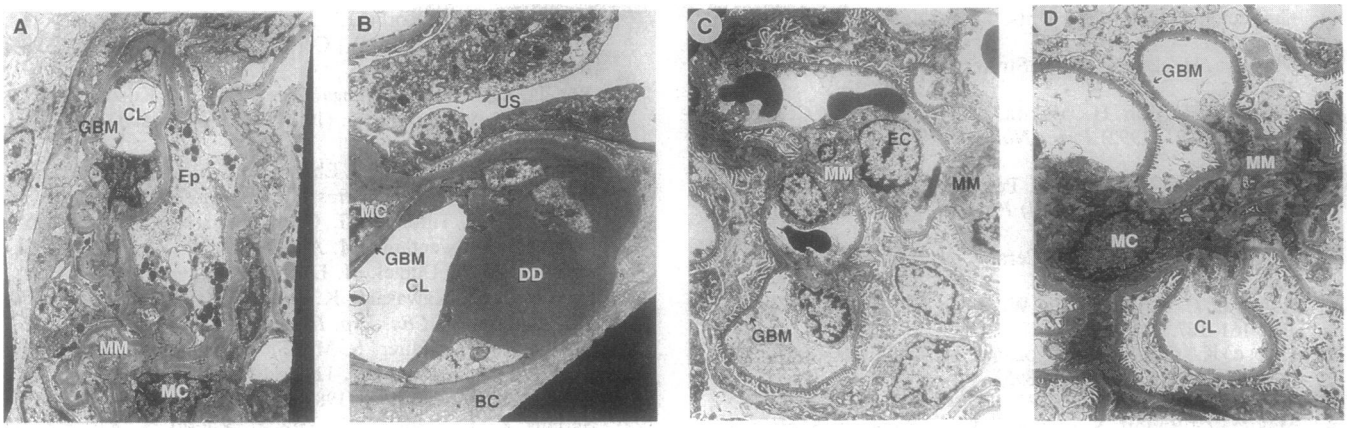

FIG. 4. Electron microscopy of AGE-RSA-treated (*A* and *B*), untreated control (*C*), and AGE/aminoguanidine-treated (*D*) rat glomeruli. (*A*, *C*, and *D*, ×970; *B*, ×2600.) EC, endothelial cell; CL, capillary loop; MM, mesangial cell; US, urinary space; Ep, epithelial cell; DD, electron-dense deposits.

per mg of protein excreted in the urine of the AGE-RSA-treated rats, compared to that found in their serum (2.5-fold above control). The increase in kidney-associated AGEs noted in AGE-RSA-treated rats can be attributed in large part to the covalent attachment of the exogenous AGE-RSA onto renal tissue through reactive moieties within it; that AGEs readily attach to collagen or circulating plasma proteins, such as low density lipoprotein, has been established recently for both *in vitro*-prepared and native AGE-peptides isolated from uremic patients (15, 16). Thus, this suggests that the chronic renal filtration of the degradation products of naturally formed AGE-proteins may lead to AGE deposition within the kidney. Consistent with this view, aminoguanidine cotreatment facilitated rapid renal clearance of AGE-peptides (8-fold above control), presumably rendered noncrosslinking.

The contribution of AGEs to glomerulosclerosis was readily apparent and unique to glomeruli from AGE-RSA-treated rats. Likewise, the protective effects of the AGE-crosslink inhibitor aminoguanidine were also marked against the glomerular hypertrophy and sclerosis, indicating that these changes were AGE-generated. Since aminoguanidine previously attenuated glomerular lesions in diabetic rats (17), this lends further support to the causal role of AGEs in diabetes. Aminoguanidine, in addition to preventing AGE-crosslinking, is also an inhibitor of the inducible form of nitric oxide synthase (18). However, there is no evidence to support such a mechanism of action in these studies, based on the absence of changes in mean blood pressures and the reported protective effect of aminoguanidine on the nitric oxide-dependent vasodilatory impairment associated either with diabetes or with AGE infusion (8). Furthermore, in the animals exposed to native RSA, there were no electron or light microscopic changes and no evidence of proteinuria attributable to the immune nephritis known to occur in connection to heterologous albumin infusion (19). This reduces the likelihood of an immune-complex-like mechanism underlying the AGE-RSA-generated lesions. These findings, although not identical, were similar to those found in experimental (streptozotocin-induced) diabetes and in certain respects, resemble the changes in human diabetic nephropathy (20).

The pattern of glomerular changes presented here also contains elements comparable to changes described in aging human and animal kidneys (2). As there can be no central role for hyperglycemia in aging-related renal injury and given the excessive accumulation of AGEs in aging tissues (4–6), it is tempting to speculate that the continuous challenge imposed on the renal filtration apparatus by circulating AGE-peptides and reactive intermediates (the natural catabolic products of tissue AGE-protein degradation) may cause renal lesions over time. Although further studies are necessary to establish this association, the model presented in these studies may prove useful in the study and prevention of aging-associated renal disease.

The glomerulosclerosis observed in these chronically AGE-treated animals may be mediated in part by the induction of cytokines and growth factors, which can regulate both cellular proliferation and protein synthesis including interleukin 1β, tumor necrosis factor α, insulin-like growth factor IA, platelet-derived growth factor, and transforming growth factor β1 (7, 9). Each of these and other factors have been implicated in the complex network controlling glomerular growth and matrix production in different experimental systems (21). As yet, however, only transforming growth factor β1 has been linked to AGE-induced glomerular changes *in vivo* (9). The cellular mechanisms underlying the effects presented here are still unclear; however, the contribution of AGE-specific receptors present on mesangial and other cells is highly probable, given that in culture these cells respond with altered expression to a similar spectrum of mediator molecules via surface AGE-binding sites (22, 23).

The present work extends the *in vivo* evidence that AGEs play an independent role in inducing glomerular hypertrophy and nephrosclerosis in normal kidneys. Thus, the presence of excessive AGEs in tissues or the circulation may critically affect the progression of diabetic and aging-related nephropathy. Moreover, this pathological pathway may be blocked by agents such as aminoguanidine, providing both a critical tool for further studies and a potential therapeutic intervention.

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