Dietary Glycotoxins

Inhibition of Reactive Products by Aminoguanidine Facilitates Renal Clearance and Reduces Tissue Sequestration

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Evidence indicates that the metabolic turnover of food-derived reactive orally absorbed advanced glycation end products (AGEs) or glycotoxins (GTs) is delayed, possibly contributing to the tissue damage induced by endogenous AGEs, especially in patients with diabetes and kidney disease. The aim of this study was to explore whether pharmacologic inhibition of dietary AGE bioreactivity by aminoguanidine (AG) can improve turnover and renal excretion of these substances. Normal Sprague-Dawley rats were fed single-labeled [^{14}C]AGE-ovalbumin, double-labeled [^{14}C-^{125}I]AGE-ovalbumin, or control ^{125}I-labeled ovalbumin diet plus free [^{14}C]glucose, with or without AG (0.2% in water). [^{14}C]AGE- and ^{125}I-labeled peptide–associated radioactivity (RA) were compared with AGE immunoreactivity (by enzyme-linked immunosorbent assay) in tissues, serum, and 72-h urine samples. The effect of AG on dietary AGE bioreactivity was assessed by monitoring the inhibition of covalent complex formation between fibronectin (FN) peptide fragments and serum components, after a meal of labeled dietary AGE with or without AG. The radiolabeled AGE diet produced serum absorption and urinary excretion peaks kinetically distinct from those of free [^{14}C]glucose or [^{125}I]ovalbumin. Some 26% of the orally absorbed AGE-ovalbumin was excreted in the urine, whereas after AG treatment, urinary excre-tion of dietary AGEs increased markedly (to >50% of absorbed). More than 60% of tissue-bound RA was found covalently deposited in kidneys and liver, whereas after treatment with AG, tissue AGE deposits were reduced to <15% of the amount found in untreated AGE-fed controls. Sera enriched for dietary GTs formed covalently linked complexes with FN, a process completely inhibitable by AG cotreatment. Amelioration of dietary GT bioreactivity by AG improves renal elimination and prevents tissue deposition of food GTs. This may afford a novel and potentially protective use of AG against excessive tissue AGE toxicity in diabetic patients with renal disease. *Diabetes* 48:1308–1315, 1999

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AG, aminoguanidine; AGE, advanced glycation end product; ANOVA, analysis of variance; AUC, area under the curve; CML, carboxymethyl-lysine; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GT, glycotoxin; HMW, high molecular weight; IR, immunoreactivity; LMW, low molecular weight; LZ, lysozyme; MG, methylglyoxal; Mr, molecular weight; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RA, radioactivity; TCA, trichloroacetic acid.

Endogenous nonenzymatic glycation of proteins and lipids by reducing sugars leads to the in vivo generation of numerous and heterogeneous moieties, advanced glycation end products (AGE) (1,2). Both the half-life and the microenvironment of a macromolecule dictate the structure and the rate of AGE formation (1,3,4), the nature of which ranges between reactive, cross-link–forming, or oxidative products (such as 1,3-deoxyglucosone [1,3-DG], methylglyoxal [MG], AP-diones [also termed glycotoxins]) and nonreactive AGEs (such as carboxymethyl-lysine [CML], pyrroles, imidazoles, and pyridines). Both classes of AGEs coexist on a given carrier polypeptide (5–7). AGEs are known to accumulate with age and glycemia and to contribute to tissue pathology by biochemical and cytotoxic pathways (3,8). Thus, the infusion into normal animals of mixtures of AGE substances produced in vitro leads to the attachment of in vivo chemically active glycotoxins (GTs) in various tissues and is followed by cell activation and overproduction of cytokines, growth factors, and extracellular matrix proteins, causing diabetes-like vascular and pathologic changes (9–11).

Steady-state serum AGE levels, which are thought to reflect the equilibrium between extravascular tissue and renally excretable pools of AGE byproducts, are shown to correlate directly with creatinine clearance and to be abnormally elevated in diabetic and nondiabetic patients with renal disease (5,12), pointing to the kidney as an important AGE-regulatory organ. Circulating bioreactive GTs that are not cleared by the kidneys may oxidatively modify or form new cross-links with plasma or tissue components such as LDL or collagen (5,6), eventually promoting peripheral tissue injury. Such events have been shown to be largely inhibitable by aminoguanidine in vitro and in vivo (5).

AGEs, long known to form in foods during heating (13–16), are also orally absorbed. Relying on an AGE-specific enzyme-linked immunosorbent assay (ELISA) (17), we have estimated that ~10% of ingested immunoreactive AGEs are transported into the circulation, two-thirds of which remain in the body—only one-third is renally eliminated within 3 days from ingestion. Advanced renal insufficiency, such as due to diabetes, was associated with a marked reduction in urinary clearance of diet-derived AGE (from 30 to <5%) (17). From that study, it was reasoned that diet-derived AGEs can impart additional adverse effects, especially in patients with diabetic renal disease. The precise influx and the potential untoward effects of dietary AGEs could not be

directly evaluated, however, since they were methodologically indistinguishable from those endogenously formed. Moreover, the findings raised the question of the need for exploring possible interventions to counteract dietary AGE toxicity.

To address these issues, we fed normal rats with single- and double-labeled AGE-modified protein diet preparations, aiming to accurately establish intestinal absorption of glycated food substances, tissue distribution, and normal kidney excretory capacity for such derivatives, together with in vivo bioreactivity. We then tested whether potentially toxic properties and renal excretion of dietary AGEs could be modulated by altering their bioreactivity using aminoguanidine (AG), for future therapeutic purposes.

RESEARCH DESIGN AND METHODS

Preparation of labeled diets and AGE assessment

Single-labeled [^{14}C]AGE-ovalbumin diet. Ovalbumin (20 mg/ml) (Sigma, St. Louis, MO) was incubated with 0.5 mol/l D-glucose and 0.5 mCi [D-^{14}C]glucose-UL (7.1 mCi/mmol) (Sigma) in 0.2 mol/l $NaHPO_4$ phosphate-buffered saline (PBS) in the presence of 0.5 mol/l EDTA at 37°C for 6 weeks, as per standard procedures (7,19,20). After extensive dialysis against PBS at 4°C, >95% of radioactivity (RA) was precipitable by trichloroacetic acid (TCA) (20%; specific activity 10^4 cpm/μg).

Double-labeled AGE diet ([^{14}C-^{125}I]AGE-ovalbumin). Aliquots of [^{14}C]AGE-ovalbumin were labeled with ^{125}I by the Iodo-Gen method (21). Samples were fractionated by Sephadex G-25M column chromatography and dialyzed against PBS until >95% of the ^{125}I-associated counts per minute was TCA-precipitable (specific activity 10^3 cpm/ng).

[^{14}C-^{125}I]AGE peptides. [^{14}C-^{125}I]-labeled AGE peptides were prepared by enzymatic proteolysis of [^{14}C-^{125}I]AGE-ovalbumin as previously described (22). Briefly, 10 ml double-labeled AGE-ovalbumin (20 mg/ml in 10 mmol/l PBS) was incubated with 2 mg proteinase K for 20 h at 37°C. Low molecular weight (LMW) peptides (<10 kDa) were then isolated by centrifugation through a Centripep-10 membrane (Amicon, Beverly, MA) for 3 h at 4000*g*. AGE peptides (500 μl of 5 mg/ml) were applied onto a 40-cm Biogel P-6 gel filtration column (Biorad, Hercules, CA), equilibrated in 10 mmol/l sodium PBS (150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 1 mmol/l sodium azide, pH 7.4), and eluted at a flow rate of 10 ml/min using the same buffer. One-milliliter fractions were collected for protein determination at 280 nm (18) and for measuring radioactivity. The void volume, estimated as the exclusion volume for hemoglobin, was 12 ml. The BioGel column was calibrated with insulin (molecular weight [Mr] 6,000), bacitracin (Mr 1,400), and glucose (Mr 180).

Control diet. The control diet consisted of ^{125}I-labeled ovalbumin (20 mg/ml) (21). Immediately before administration of iodinated ovalbumin, [^{14}C]glucose (~2×10^6 cpm) was added as a tracer for unreacted glucose-derived counts per minute.

AGE content. The AGE content in the diet preparations was determined by AGE-ELISA, using previously characterized anti–AGE-RNase polyclonal and monoclonal antibodies (19,20) AGE-ovalbumin, 300 AGE U/μg, and ovalbumin, 2.5 AGE U/μg. The presence of known AGEs, such as MG and CML, in diet preparations was confirmed by the use of additional anti-MG monoclonal antibody (23) (kindly provided by Dr. M. Brownlee, Albert Einstein School of Medicine, New York) and anti-CML monoclonal antibody 6D12 (Waco, Osaka, Japan) (20). AGE diet contained 267 U MG or 370 U CML per milligram, and control ovalbumin, 18 U MG and 3.0 U CML per milligram.

Monitoring of dietary AGE transit and activity

AGE radioactivity. RA in serum, urine, and tissues was measured using the specific window for either ^{125}I or ^{14}C. To count ^{14}C in tissue pieces, the samples were homogenized, dissolved in BTS-450, decolorized with 30% H_2O_2, and counted after adding glacial acetic acid and Ready Organic (Beckman, Fullerton, CA). The data were expressed in counts per minute per 100 μl for serum, counts per minute per gram for tissue, and counts per minute per hour for urine samples.

AGE immunoreactivity. AGE immunoreactivity (IR) in serum, urine, and tissues was routinely measured by a competitive AGE-specific ELISA based on a monoclonal anti-CML antibody (20), with minor modifications. Before the assay, serum samples were diluted 1:5 in PBS and digested with 0.1% (wt/wt) proteinase K (Sigma) at 37°C overnight. Proteinase K was inactivated at 70°C for 1 h. Serum data were expressed as AGE units per milliliter. Urinary AGE levels were expressed as AGE units excreted per hour.

AGE/lysozyme-binding activity. Lysozyme (LZ) is able to bind specifically to reactive AGEs (7,24). Chicken LZ (Sigma) covalently bound to Sephadex (LZ-column) was previously used to remove non-AGE components from serum (7). Pooled serum samples adjusted for radioactivity level (~5×10^6 cpm/μg) were diluted 1:5 with PBS and passed over such an AGE-affinity matrix. After the void volume, the LZ-bound components were eluted with 0.1 N NaOH and immediately neutralized with 6 N HCl. Consistent with previous evidence (7), residual radioactivity on LZ-matrix was <3% for all samples. Radioactivity was determined in the AGE-enriched fractions (5 mg/ml).

AGE cross-linking activity. Serum aliquots were incubated with fibronectin (FN) fragments (100 μg/ml) (Sigma) in the presence or absence of the AGE inhibitor aminoguanidine (1%) and proteinase inhibitors at 37°C for 72 h. High-molecular-weight (HMW) complexes were separated from LMW fractions by using either Centriprep-10 (Amicon) or gel filtration (Biogel), and AGE-specific radioactivity was determined in both fractions. The fractions obtained before and after incubation with FN were electrophoresed, visualized on autoradiographs, and analyzed by densitometry.

Experimental diet design. To assess dietary glycotoxin influx, the following absorption studies were performed using 6-month-old, healthy, female Sprague-Dawley rats (six rats per group, kept according to the Institute's guidelines for care and use of laboratory animals). Each of the rats received *1*) the equivalent of 2×10^6 cpm (either ^{14}C or ^{125}I) of AGE diet ([^{14}C-^{125}I]AGE-ovalbumin) or the same counts per minute of control diet ([^{125}I]ovalbumin mixed with [^{14}C]glucose) by oral gavage; *2*) 2×10^6 cpm of [^{14}C]AGE ^{125}I-labeled peptides or the same amount of control [^{125}I]peptides mixed with [^{14}C]glucose by intravenous injection (10 μg/20 μl); and *3*) AG-HCl (0.2% in drinking water), for 3 days before the test preparation and for the course of the study. For the above experiments, rats were kept in metabolic cages with food and water provided ad libitum. Blood and urine samples were collected at various intervals for up to 72 h after each test.

For assessing tissue distribution of dietary GTs, animals were exposed to labeled AGE or control diet in drinking water for 5 days, followed by a 3-day washout period before being assayed. One group of rats was treated with AG 3 days before the start of a test AGE or control diet and until being assayed. At the end of each experiment, rats were anesthetized in a CO_2 chamber, blood samples were collected, and rats were perfused with PBS to remove remaining blood before organ retrieval. Kidneys, heart, lungs, pancreas, spleen, and liver were saved for determining RA.

Calculations and statistics. Total AGE diet absorption in serum was calculated using a standard formula (trapezoidal) (26). Briefly, serum AGE-associated radioactivities were plotted against time, and the area under the curve (AUC_{t-t2}) was integrated according to $S = (t_2 - t_1)/2 \times (C_2 + C_1)$, where S is total, t_1 and t_2 are levels of administered agent at time points 1 and 2, and C_1 and C_2 are baseline levels at the corresponding time points. The results were expressed in counts per minute per hour per milliliter. Total absorption was obtained by multiplying these values by total blood volume. Total urinary AGE excretion was defined as the sum of AGE-associated RA of urine samples collected from each animal over 72 h and was expressed as counts per minute per hour. Comparisons of the means of serum AUCs or total urinary AGE excretion among different groups were based on one-way analysis of variance (ANOVA) with post-hoc comparison by the Student-Newman-Kuel's test. Comparisons between AGE diet– and control diet–induced changes in serum and urine AGE levels were based on one-way ANOVA.

RESULTS

Serum influx and urinary excretion of labeled dietary AGEs in the rat

[^{14}C]AGE RA and AGE IR. Within 1 h of feeding a single dose of double-labeled AGE diet ([^{14}C-^{125}I]AGE-ovalbumin), serum levels of [^{14}C]AGE-specific RA and IR began to rise gradually, peaked within 10 h, and returned to baseline levels by 20 h (Fig. 1*A* and *C*), compared with the early but brief rise produced by the control diet ([^{125}I]ovalbumin) (Fig. 1*A* and *C*), which was without notable IR activity despite the free [^{14}C]glucose added as a tracer (Fig. 1*E*). At 8–10 h after feeding of the AGE diet, we detected a 3-fold rise in MG-like IR and a 2.5-fold rise in CML-like IR above baseline. Urinary AGE diet RA and IR also increased in tandem within 1 h, peaked within 6 h, and returned to baseline levels by 24 h (Fig. 1*B* and *D*); only traces of IR were found in urine from rats given control diet plus [^{14}C]glucose (Fig. 1*B* and *F*). The concordance between AGE RA (^{14}C) and AGE IR indicated that tracing of ^{14}C-RA could be relied on as a means of monitoring intestinal influx and turnover of orally supplied [^{14}C]AGE diets and not of unreacted glucose or of unmodified protein peptides. The total intestinal absorption of dietary AGEs, based on the AUC of ^{14}C counts per minute found in the blood, was estimated at ~10% of the total ingested AGE, whereas the proportional AGE excreted in

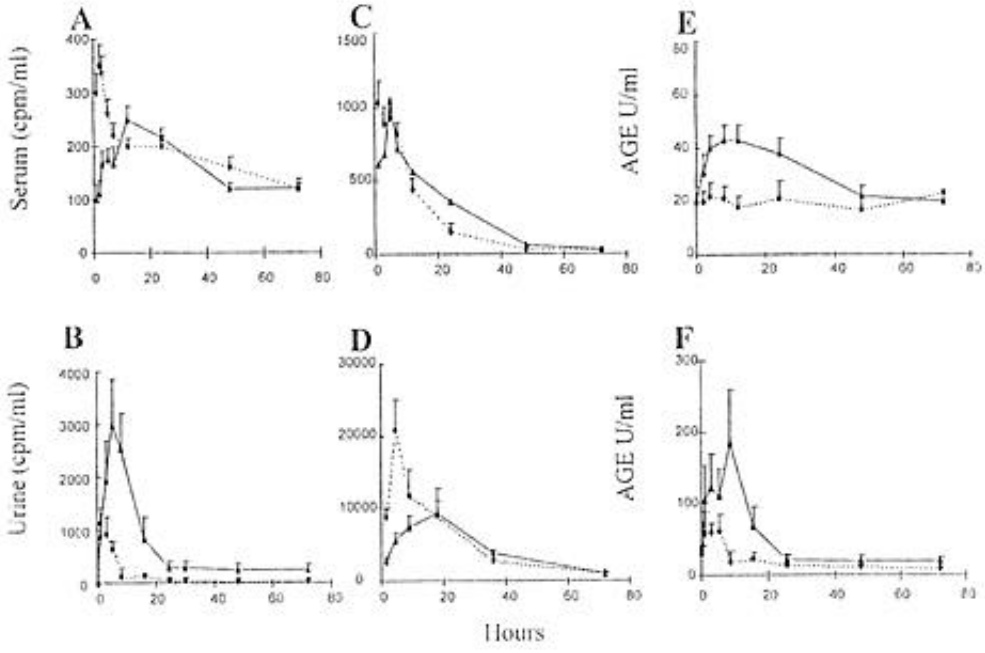

FIG. 1. Serum influx and renal excretion kinetics of dietary AGE. A single dose of AGE diet ([^{14}C-^{125}I]AGE-ovalbumin) or control diet ([^{125}I]ovalbumin plus [^{14}C]glucose) was fed to normal rats (n = 5 per group) by gavage. Serial serum samples (100 µl) (*A*, *C*, *E*) and total 72-h urine (*B*, *D*, *F*) were collected. AGE levels were determined as ^{14}C-plus ^{125}I-associated radioactivity or as AGE units. *A* and *B*: ^{14}C counts per minute. *C* and *D*: ^{125}I counts per minute. *E* and *F*: AGE units. —, AGE diet; - - -, control diet. Data points represent mean ± SE values from all animals.

the urine within a period of 3 days was equal to 30% of that found in serum, or about 3% of the ingested amount of AGE diet.

^{125}I-derived radioactivity. The amount of ^{125}I-derived RA transported into serum was similar for both AGE and control diets, with a 2-h lag time between peaks ([^{125}I]ovalbumin at 6 h and [^{125}I]AGE-ovalbumin at 8 h) (Fig. 1*C*). In contrast, the proportion of ^{125}I-associated counts per minute derived from the AGE diet excreted in the urine appeared significantly lower than that derived from the control diet (Fig. 1*D*). Based on AUC estimates, levels of ^{125}I-labeled AGE-derived counts per minute excreted in the urine were lower than those of the control (26 vs. 50% of counts per minute in serum, respectively) and consistent with the ^{14}C-labeled AGE diet–derived counts per minute.

Effect of AG coadministration on influx and excretion of dietary AGEs. In serum, AG administration did not alter the kinetic profiles of either AGE levels (Fig. 2*A* and *C*) or control diet (not shown), whether measured as [^{14}C]AGE or as [^{125}I]AGE counts per minute. In contrast, during cotreatment with AG, the AGE diet–derived urinary excretion peaks appeared earlier and with larger AUCs than those derived from control diet–fed rats (Fig. 2*B* and *D*). Furthermore, coadministration of AG induced an increase in the fractional excretion of [^{14}C]AGE and [^{125}I]AGE counts per minute to a level that was equal to the excretion seen in control diet–fed animals (from ~26 to ~50%, $P < 0.005$), having no such effect on the urinary excretion of control diet substances (Fig. 3*A* and *B*).

Urinary excretion of predigested AGE peptides infused intravenously. To explore whether normal rat kidneys exhibit a restricted excretory capacity specific for AGE-modified peptides and amino acids, compared with unmodified ones, we compared serum kinetics and urinary excretion of predigested ^{14}C- or ^{125}I-labeled AGE-modified or native ovalbumin peptides, given as a single intravenous injection.

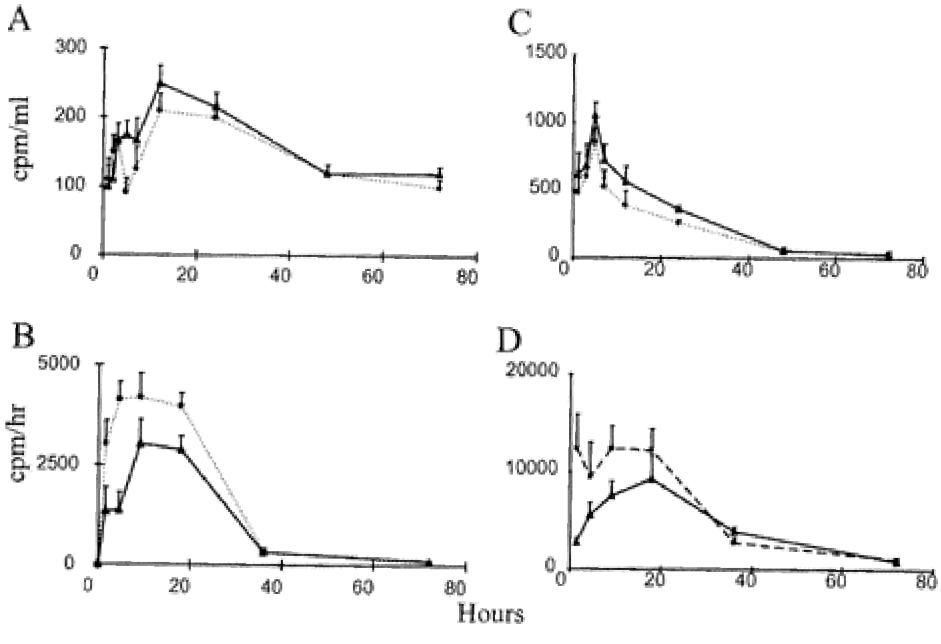

FIG. 2. Effect of AG on the kinetics of dietary AGE absorption and urinary excretion. Animals (n = 5 per group) were exposed to AG in drinking water (0.2% for 3 days) and then fed a single dose of double-labeled AGE or control diet, as described in Fig. 1. Serum and urine samples were collected at the indicated intervals for ^{14}C and ^{125}I determination over 72 h. Serum (*A*) and urine (*B*) kinetics of ^{14}C radioactivity and serum (*C*) and urine (*D*) kinetics of ^{125}I radioactivity are shown. —, AGE diet; - - -, AGE diet plus AG. Data represent mean ± SE values.

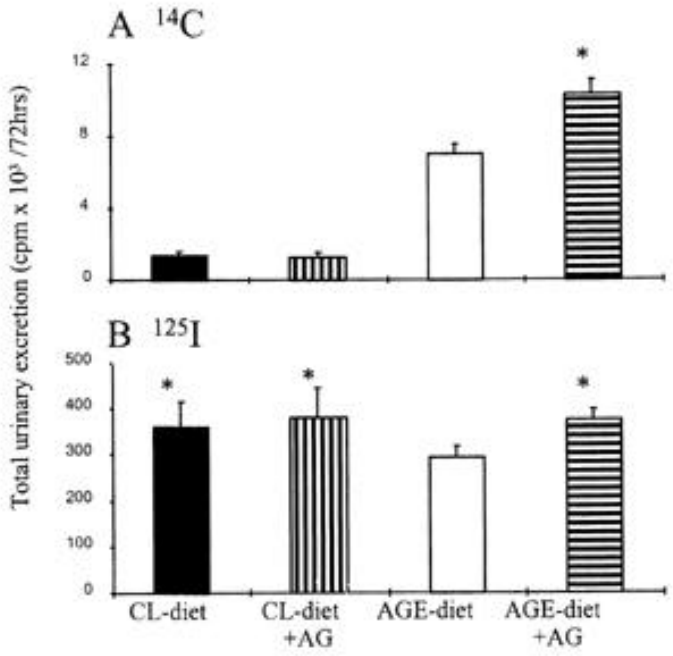

FIG. 3. Aminoguanidine enhances urinary excretion of orally absorbed AGEs. Urinary excretion of double-labeled dietary AGE and control diet (CL-diet) was determined over 72 h as described in Fig. 1. *A*: ^{14}C counts per minute; *B*: ^{125}I counts per minute. Data are expressed as total counts per minute per 72 h. **P* < 0.05 compared with the AGE diet group, *n* = 5 rats per group.

Ultrafiltered serum fractions containing LMW AGE or control peptides (Mr ~2–6 kDa) were pooled for further study.

[^{14}C]AGE-associated radioactivity. The intravenous injection of [^{14}C]AGE-ovalbumin peptides produced serum peaks within a few minutes, which declined rapidly to background levels (Fig. 4*A*). Similar to orally supplied AGEs, only 30% of intravenously injected AGE-ovalbumin peptides were eliminated by the kidney within 72 h, whereas only traces of free [^{14}C]glucose, injected as control, were found in the urine (Fig. 4*B*).

[^{125}I]AGE-associated RA. The infusion of either [^{125}I]AGE or control ovalbumin peptides induced rapid elevations of serum RA, similar to those observed with [^{14}C]AGE preparations (Fig. 4*C*). Interestingly, AGE peptides produced a far smaller urinary AUC than that of unmodified peptides (Fig. 4*D*). The total amount of [^{125}I]AGE peptides excreted during the 72-h period tested was equivalent to 25–30% of that present in serum, compared with 50% of unmodified peptides excreted.

Effect of AG on tissue distribution and accumulation of dietary AGEs. Rats were administered a double-labeled [^{14}C-^{125}I]AGE diet or control ^{125}I diet, with or without AG (0.2% in water) for 5 days, followed by a period of 3 days feeding on regular rodent food and water ad libitum. Tissue accumulation of dietary AGEs was assessed by measuring ^{14}C and ^{125}I RA in selected organs.

[^{14}C-^{125}I]AGE-ovalbumin RA. Since only major organs were selected for RA content, the total counts per minute retained in the entire tissue compartment was not determined. Among the tissues examined, kidneys and liver contained the highest levels of ^{14}C counts per minute per gram of tissue (>60% combined), presumably AGE-derived (Fig. 5*A*). Coadministration of AG was associated with significantly lower levels of radioactivity in most tissues (to <15% of non–AG-treated tissues), whereas it had no effect on the generally low ^{14}C counts per minute level found in tissues from rats fed control diet (ovalbumin) and [^{14}C]glucose, demonstrating that most of the radioactivity deposited in the tissues was AGE-specific and covalently bound. In the rats fed the [^{14}C]AGE diet, after the 3-day washout period, only a modest level of counts per minute was present in the circulation (~5%) compared with the large proportion of RA distributed to the tissues. Almost the entire amount of circulating counts per minute was found in the HMW (>10 kDa) serum fraction.

[^{125}I]AGE-ovalbumin–derived RA. The tissue distribution of [^{125}I]AGE-ovalbumin was similar to that of [^{14}C]AGE-ovalbumin (Fig. 5*B*). The inhibitory effect linked to coadministration of AG was also similar. Control [^{125}I]ovalbumin-derived RA was also observed in tissues, but at much lower levels, and it was not inhibitable by AG.

Diet AGE recognition by soluble AGE-binding proteins. Lysozyme has been shown to recognize and specifically bind AGE epitopes with high affinity (7), and this property has been used to remove AGEs from human serum (7,24).

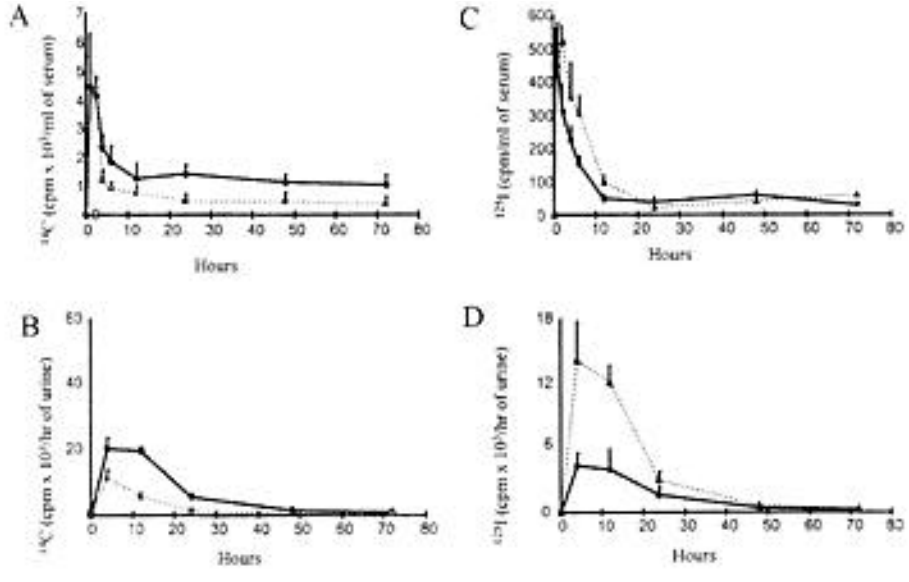

FIG. 4. Renal excretion of circulating AGE peptides is low compared with that of native peptides. A single dose of double-labeled predigested AGE-modified or native ovalbumin peptides was injected into rats (10 µg/20 µl; *n* = 5 per group). Serum (*A*) and urine (*B*) profiles of ^{14}C counts per minute and serum (*C*) and urine (*D*) profiles of ^{125}I counts per minute are shown. —, AGE-ovalbumin; - - -, unmodified ovalbumin. Data represent mean ± SE values.

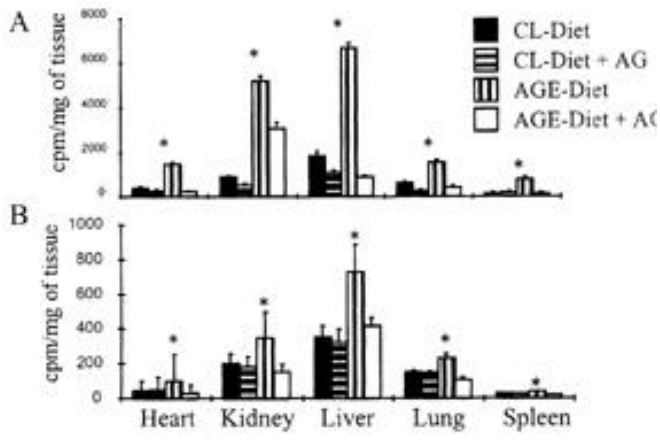

FIG. 5. Tissue deposition of orally absorbed AGEs is prevented by aminoguanidine. Rats received double-labeled AGE diet or control diet (CL-diet) with or without AG (0.2% in drinking water) for 5 days, followed by 3 days of plain water. After extensive perfusion with PBS, organs were collected, and tissue-associated radioactivity was determined. *A*: ^{14}C counts per minute. *B*: ^{125}I counts per minute. Data are expressed as the mean ± SE counts per minute per gram of tissue from five rats per group. **P* < 0.05 compared with the corresponding AG-treated group.

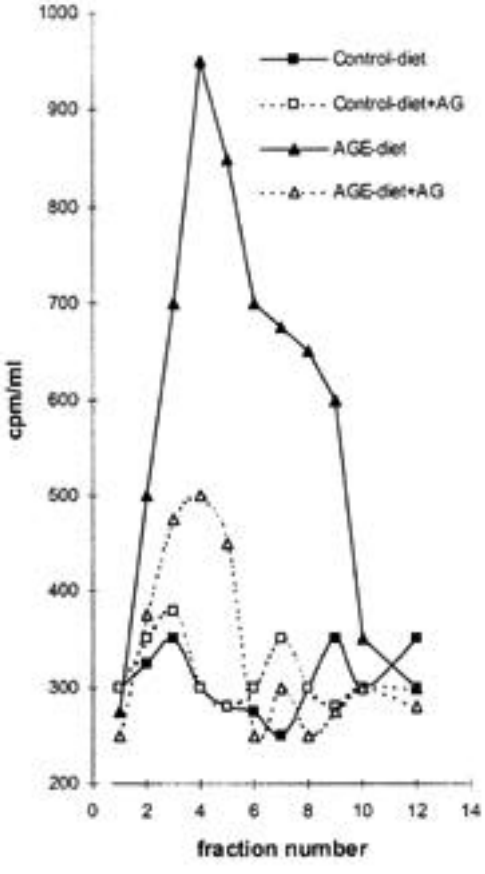

FIG. 6. Aminoguanidine diminishes availability of dietary AGE in postabsorptive sera. Rat serum samples collected between 6 and 24 h after a single dose of double-labeled AGE (△, ▲) or control (□, ■) diet, fed with (△, □) or without (▲, ■) AG (0.2% in water), were passed through an AGE-affinity LZ-column as described. Total ^{14}C counts per minute and ^{125}I counts per minute were determined in the eluted fractions. Data are representative of three independent experiments.

When pooled sera obtained within 6 h of ingestion of labeled AGE diet or control diet were passed through and eluted from an LZ-column, a sevenfold higher amount of radioactivity was retrieved compared with that obtained from rats fed a control diet ($P < 0.005$) (Fig. 6). This finding suggested that diet-derived AGE intermediates transported in the bloodstream include epitopes recognized by and bound reversibly onto LZ, not unlike other model AGEs (7,24). In contrast, elution fractions from rats cotreated with AG yielded a fourfold lower amount of LZ-bound RA compared with that of AGE sera ($P < 0.05$) (Fig. 6); with regard to the control sera, >90% of counts per minute was released in the void volume (not shown).

In vivo bioreactivity of dietary [^{14}C]AGEs. To confirm whether a subgroup of dietary AGEs, after intestinal digestion and transport into the bloodstream, retained bioreactivity on interaction with serum or tissue components, we incubated double-labeled AGE diet serum samples, obtained 6 h after ingestion, with unmodified FN at 37°C for 72 h. HMW aggregates containing [^{14}C]AGE radioactivity were formed in these mixtures, a result that was ameliorated by the addition of AG (Fig. 7*A*). There were no aggregates formed in samples containing control diet serum. When post–AGE diet sera from rats cotreated with AG were incubated with FN, a major portion of the [^{14}C]AGE counts per minute remained in the LMW fraction, indicating that HMW complex–forming reactive moieties were drastically reduced or inactivated compared with non–AG-treated rats (Fig. 7*B*). This finding was further confirmed by adding [^{14}C]AGE diet–enriched serum fractions to native RNase and observing dimer and trimer formation, a covalent activity completely prevented by AG, using immunoblot and autoradiography (data not shown).

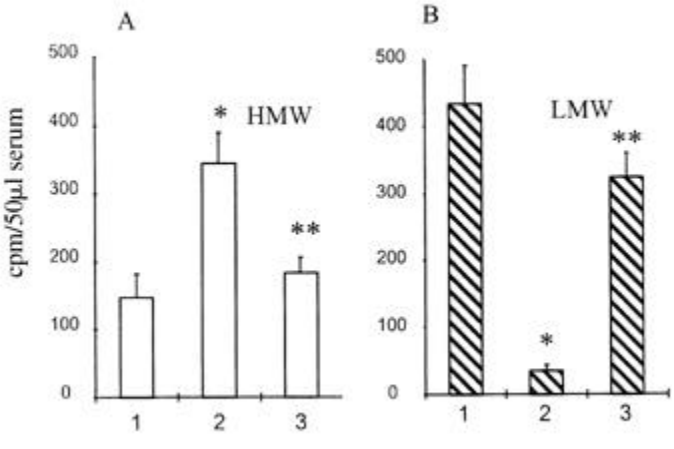

FIG. 7. Aminoguanidine normalizes the increase of circulating diet-derived GTs. Postabsorptive serum samples, collected between 6 and 24 h after feeding a single dose of [^{14}C]AGE-ovalbumin diet with or without AG (0.2% added to water), were incubated with fibronectin fragments for 72 h at 37°C. HMW (*A*) and LMW (*B*) fractions were separated by ultracentrifugation as described. Total ^{14}C counts per minute was determined in both fractions. 1, AGE sera before incubation with FN; 2, AGE sera after incubation with FN; 3, AGE and AG sera after incubation with FN. Data represent means ± SE of three identical experiments. **P* < 0.01 compared with values at preincubation with FN; ***P* < 0.01 compared with values obtained in the absence of AG.

DISCUSSION

The present animal study demonstrates that two cardinal features of orally absorbed AGEs, prolonged metabolic turnover and delayed renal elimination, are largely due to the presence of bioreactive compounds, also termed glycotoxins, as evidenced by the effective reversal of these properties by aminoguanidine administration.

The pathogenic effects of the bioreactivity exerted by endogenously formed glucose-derived AGEs have been investigated for the last 20 years (1–4). Of relevance has been the recent recognition that glycoxidation intermediates released during the catabolic breakdown of tissue AGE components represent an important source of tissue toxicity. These substances are apparently capable of propagating covalent cross-link formation or oxidative modification of serum and tissue proteins, such as LDL, α2-microglobulin, and collagen (5,6,25). A significant portion of glycotoxin-related pathology is currently attributed to the kidney, the decline in function of which results in a dramatic reduction of the renal clearance of AGE byproducts (5,12) and a corresponding enlargement of the total-body AGE pool.

That heat treatment of common foods usually rich in carbohydrates vastly accelerates nonenzymatic glycation, or "browning," has been well known (13). The consequences of the constant influx of browning products, however, were viewed neither in the context of their bioreactivity nor in relation to specific organ function and therefore were not looked upon as a serious health hazard, until in a recent study we suggested that orally absorbed food glycotoxins are excreted by the human kidneys only in part and less so by diseased kidneys (17). Though based on a sensitive AGE-specific immunoassay (19,20,27), that study was limited by an inability to distinguish between ingested AGEs and those endogenously formed. Likewise, the bioreactivity and potential toxicity of food glycotoxins could not be ascertained accurately and independently. More importantly, further steps aiming at modulating their putative toxicity could not be taken.

In this report, to overcome these problems, we combined immunoreactivity with radioactivity measurements of double-labeled standardized diet preparations based on a mostly single protein source, egg white or ovalbumin. Previous evidence (17) indicated a >10-fold increase in AGE content during the heating of ovalbumin with sugar (as in meringue). An even greater increase in AGE content was estimated when lipid-rich foods were heat-prepared in the presence of carbohydrates (>100-fold) (17), presumably due to the synergistic effects of heat- and glucose-catalyzed lipid oxidation (6). Complex foods used in daily life are more heavily modified than the single-protein preparations used in the present study at minimal doses. Thus, the data presented are likely to underestimate the actual daily influx of glycotoxins. Also, among the vastly heterogeneous classes of dietary AGEs, only two known structures, the highly reactive MG and the terminal nonreactive glycoxidation end product CML, were sought out with the help of specific antibodies, both in the diet and in postprandial sera; many others remain elusive. Thus, it remains unclear how many GT compounds are absorbed and what are the prevailing structures among them. Based on the internal consistency between [^{14}C]AGE RA and AGE IR kinetic data in serum and urine, however, the former approach proved more sensitive and reliable for distinguishing influx and turnover of dietary glycotoxins from endogenous AGE. In addition, the inclusion of free radioactive glucose as a tracer enabled us to compare diet-derived glycotoxins with unreacted free glucose. The inclusion of ^{125}I-labeled peptides permitted the observation of the fate of AGE-moieties separately from that of small peptides normally absorbed from the intestine.

Consistent with our initial studies in humans (17), ~10% of the ingested labeled AGEs were absorbed into the rat circulation, and only ~30% of that was excreted in the urine within 72 h. The latter was further confirmed by determining the urinary excretion of a precisely quantitated amount of predigested labeled AGE peptide preparation, administered intravenously: ~70% of it remained in the body beyond the 3 days of observation. The quantitative discrepancy between postprandial and postabsorptive glycotoxin availability in serum and eventual retrieval in the urine led to the speculation of a markedly restricted and/or delayed urinary excretion pattern specific to glycotoxins. This intriguing observation, while in need of further exploration, lends itself to several interpretations.

For instance, it is reasonable to expect that exogenous AGEs, once in circulation, are quickly dispersed into the extravascular space, readily engaging in complex interactions with tissue and cell components. Also, dietary AGEs could be largely secreted and reabsorbed in the proximal tubule, as previously speculated (28). The precise pathway of AGE handling by the excretory apparatus of the kidney is not completely understood. However, to the extent that covalent cross-link–forming reactivity is a prominent property of AGE-modified substances, the evidence presented herein strongly supports the notion that food-derived glycotoxins possess a similar reactivity against multiple sites, especially kidneys, liver, and heart (13–16,29). With regard to the nature of this interaction, it would appear to be largely covalent, owing to the presence of reactive glycotoxins such as MG in the mixture of diet AGEs (13,14,30,31). Because of the highly unstable nature of MG-like products, only a limited number of moieties are likely to exist in an unreacted state, the latter exemplified by the appearance of HMW complexes. Indeed, compared with tissues from animals fed a control diet or glucose alone, ^{14}C-associated radioactivity was significantly higher in animal tissues exposed to the AGE-modified diet and was inhibitable by aminoguanidine. This is consistent with, and supported by, several studies in animals infused with mixtures of AGE-rich preparations and aminoguanidine (9–11). Thus, the transfer of cross-linking AGE-activity into serum is of particular significance, since it defines in part the potential for glycotoxin toxicity contained in complex cooked foods, another aspect being the capacity for glycoxidative damage (1–3). Further studies are required to elucidate the chemical structure of prominent reactive glycotoxins in foods.

Dietary AGEs could also in part be subject to receptor-mediated uptake in a variety of tissues (21,22,29,32). In this context, the intriguing possibility of dietary AGE–related cellular activation leading to stimulation of cytokines and growth-promoting factors, as is shown to occur by endogenous or preformed AGEs (3,4), also raises the possibility of additional modes of toxicity, not directly addressed herein.

The question of diet GT–related toxicity was focused here on the potential for covalent intermolecular complex formation. This was probed further with the aid of a novel method, using lysozyme's high AGE-binding affinity (24). This approach was used previously to deplete excess AGEs

from diabetic/uremic sera (7). In the present studies, the presence of LZ-recognizable dietary AGEs in serum suggested possible structural similarity between exogenous and endogenous AGE species, namely CML and MG. Furthermore, the dietary glycotoxins eluted off the LZ-matrix appeared to include bioreactive species. Indeed, when [^{14}C]AGE-enriched serum samples were incubated with native FN peptide fragments, the proportion of ^{14}C-radioactivity in the resulting HMW fractions rose significantly and did so in an AG-inhibitable manner, indicating that exogenous reactive glycotoxins were present in the postabsorptive sera. These substances could act as bridging elements between or within polypeptide segments, causing permanent conformational changes and functional damage.

In addition to their chemical properties, infused glycotoxins are capable of inducing growth-promoting genes (3,10), possibly accelerating athero- and glomerulosclerosis; these effects were also largely prevented by AG (9–11). Although not directly studied here, the evidence from such studies was compelling enough to consider ways of interfering with the bioreactivity of oral GTs. This in turn might lead to a reduction in their transit time, minimizing cellular toxicity. In this context, the significantly reduced level of serum GTs in rats cotreated with AG could be explained by the enhanced urinary excretion of GT, indeed suggesting a shortened transit time for these substances once rendered inactive by AG.

AG is known to prevent endogenous AGE-induced tissue damage in numerous animal models by several mechanisms (3,4,6,27,33–37), a major one of which is the inhibition of post-Amadori reactive intermediates, including "reactive" cross-link–forming and oxygen radical–generating ones, as proposed previously (23,33,38). Our data herein suggest that AG can also prevent tissue accumulation of dietary reactive GTs, probably through an inhibitory effect on cross-link formation between food GTs and tissues. This AG effect may explain the enhanced urinary excretion of food GTs, as AG can prevent their sequestration into tissues, or else it could be due to enhanced secretion or reduced reabsorption of AGE moieties by as yet unverified mechanisms.

In summary, dietary AGEs contain bioreactive molecules likely to affect many tissues, largely the kidney, because of the lifelong massive traffic of these toxins through that organ. Inhibitors of AGE reactivity, such as AG, by preventing tissue sequestration and by enhancing urinary elimination of dietary GTs, may provide significant long-term tissue protection. At the same time, these findings raise clinically relevant questions in view of the universal population exposure to these substances.

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