
Original Articles

Lysozyme Enhances Renal Excretion of Advanced Glycation Endproducts In Vivo and Suppresses Adverse AGE-mediated Cellular Effects In Vitro: A Potential AGE Sequestration Therapy for Diabetic Nephropathy?

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Abstract

Background: Lysozyme (LZ), a host-defense protein, contains an 18 amino-acid domain with high affinity binding for sugar-derived proteins or lipids, called advanced glycation endproducts (AGE), that are implicated in diabetes- and age-dependent complications (DC).

Materials and Methods: A) The effects of LZ on AGE-removal were tested in vivo. LZ was injected (200 ug/day, i.p., ×2 weeks) in non-obese diabetic (NOD), *db/db* (+/+) mice, and non-diabetic, AGE-infused Sprague-Dawley rats. B) LZ: AGE interactions with macrophage-like T1B-183 cells (Mf) and mesangial cells (MC) were tested in vitro.

Results: A) In NOD mice, LZ reduced the elevated basal serum AGE (sAGE) ($p < 0.05$), enhanced urinary AGE (uAGE) excretion by ~2-fold ($p < 0.01$), while it reduced albuminuria (UA), $p < 0.005$. In *db/db* mice, LZ infusion

also reduced the elevated sAGE ($p < 0.05$), doubled uAGE excretion ($p < 0.05$), and decreased UA ($p < 0.01$). In addition, LZ maintained normal sAGE in normal rats infused with AGE-BSA, as it doubled the urinary AGE (uAGE) clearance ($p < 0.01$). B) LZ stimulated the uptake and degradation of ^{125}I -labeled AGE-BSA and ^{251}I -human serum AGE by Mf, while suppressing AGE-induced TNF α and IGF-I production. In MC, LZ suppressed the AGE-promoted PDGF-B, $\alpha 1$ type IV collagen, and tenascin mRNA levels, and restored the AGE-suppressed expression and activity of MMP-9, but not MMP-2.

Conclusion: LZ may act to: a) accelerate renal *in-vivo* AGE clearance, b) suppress macrophage and mesangial cell-specific gene activation in vitro, and c) improve albuminuria due to diabetes. These data suggest that LZ by sequestering AGEs may protect against diabetic renal damage.

Introduction

Advanced glycation endproducts (AGE) are a heterogeneous group of compounds derived from the non-enzymatic interaction between reducing sugars and proteins (1,2). The formation of AGE is a ubiquitous biochemical process accompanied by the generation of reactive oxygen species (ROS) (3,4). Over the past twenty years, the pathogenicity of AGE has been extensively studied, especially in connection with diabetes and age-related diseases that are associated with elevated levels of AGE production. Certain reactive AGE precursors, such as methylglyoxal (MG), 1,3-deoxyglucosone, and protein-linked Amadori product-dione, have been identified as potentially pathogenic intermediates in tissue injury (1,2). AGE

precursors can react with serum or tissue components and, with the production of oxidative species, directly alter the physical and structural properties of tissue components (5-8). In addition, AGE elicit a wide range of cell-mediated responses, thereby inducing phenotypic changes that lead to reactive oxygen species (ROS) generation, inflammatory cell activation, and growth factor/cytokine production (1,3,9, 10), often via AGE-specific receptors (3,9). Under conditions of excessive circulating or tissue AGE levels, as in diabetes, aging, and renal insufficiency, AGE-mediated pathologies are more pronounced. Inhibition of AGE accumulation, through anti-AGE agents (11-13) or via dietary AGE restriction (14,15), prevents renal and vascular abnormalities in diabetic and aging animals, confirming the causal role for AGE in tissue damage and in diabetic pathologies.

Lysozyme (LZ) is a well-characterized, naturally occurring protein that exerts anti-microbial effects through the catalytic degradation of the peptidoglycan

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components of the bacterial cell wall (16). We have recently reported the previously unrecognized ability of lysozyme to bind AGE with high affinity ($K_d = 50$ nM) (17,18). The AGE-binding site was mapped to a 17-amino acid hydrophilic domain, bounded by cysteines, located within one of the two LZ catalytic regions. This AGE-binding cysteine-bounded domain, termed the ABCD loop (17), was shown to bind AGE species including tissue-reactive derivatives found in circulation (18), suggesting that LZ could be used for the capture, sequestration and disposal of toxic AGE formed *in vivo*.

In this study, we investigated the *in-vivo* effects of hen egg LZ on serum AGE (sAGE) clearance and renal excretion under normal and diabetic conditions. In addition, we have begun to explore the cellular mechanism of LZ action, with particular emphasis on macrophage and mesangial cell AGE-activation.

Materials and Methods

Reagents

Hen egg white lysozyme (LZ), D-glucose, cyanogen bromide-activated Sepharose 4B, and p-nitrophenyl phosphate tablets, glyoxylic acid, methylglyoxal and NaBH_3CN were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-hen egg lysozyme antisera were from Biodesign (ME, USA). Sodium ^{125}I -iodide was purchased from DuPont-Merck Pharmaceutical Co. (Wilmington, DE, USA). Low-endotoxin bovine serum albumin (BSA) (Sigma Chemical Co.) was passed sequentially over an Affi-Gel Blue column (Bio-Rad Laboratories, Hercules, CA, USA), a heparin-Sepharose CL6B column (Pharmacia, LDB, Uppsala, Sweden), and an endotoxin-binding affinity column (Pierce, Rockford, IL, USA) to remove various contaminants. BSA prepared in this manner then was incubated with or without 0.5 M D-glucose in 0.2 M phosphate buffer (pH 7.4) at 37°C , for 4 weeks under sterile conditions. Low molecular weight reactants and free glucose were removed by dialysis against phosphate buffered saline (PBS), as previously described (19). AGE levels, measured by an anti-AGE monoclonal antibody that recognizes CML-like epitopes (20) were: AGE-BSA, 250 U/mg protein; unmodified BSA, 0.9 U/mg protein. Methylglyoxal- (MG) and N^ϵ -carboxymethyllysine (CML)-modified BSA were prepared according to previously published procedures (21,22). Briefly, MG-BSA was prepared by incubating BSA (20 mg/mL) with 150 mM MG in the presence of NaBH_3CN (0.45 M), in PBS (0.1 M, pH 10.0), followed by overnight dialysis against PBS at 4°C . CML-BSA was prepared by incubating BSA in 0.2 M PBS, pH 7.8, containing glyoxylic acid (0.15 M) and NaBH_3CN (0.45 M) for 24 h at 37°C (22). BSA derivatives were dialyzed and kept frozen at -80°C . GC-MS analyses indicated that MG-BSA contained 22.4 modified arginine and 10 modified lysine residues/molecule, and CML-BSA contained 21 modified lysines/ molecule. Aliquots of

each protein preparation, as well as of human diabetic sera (see below), were iodinated using Iodo-Beads (Pierce). After TCA (20%) precipitation, $\sim 95\%$ of ^{125}I was protein-bound (specific activity: $\sim 0.8 - 1.0 \times 10^3$ cpm/ng of protein). $\text{TNF}\alpha$ and IGF-I were determined by a mouse $\text{TNF}\alpha$ immunoassay kit (Biosource International, Camarillo, CA, USA) and a rat IGF-I immunoassay kit (Diagnostic System Laboratory Inc, Webster, TX, USA), respectively.

Isolation of Human Serum AGE-proteins

Human serum samples were obtained from 10 diabetic patients with advanced renal insufficiency (age: 60 ± 8 , years of diabetes: 16 ± 4 , HbA1c: 7%), stored at -80°C , and used as a source of *in-vivo-formed* AGE-proteins. Human serum AGE-proteins were enriched by the use of an AGE-affinity column prepared as previously described (18). Briefly, lysozyme was conjugated to cyanogen-bromide-activated Sepharose 4B beads. Samples were diluted 1:5 with PBS just before loading on the column (bed volume, 2 ml). After collecting pass-through fractions, columns were washed and bound fractions (0.5 ml/fraction) were eluted with 0.1N NaOH, and neutralized immediately with HCl; protein concentration was determined by BCA-protein analysis (Pierce). AGE levels were determined by ELISA (19,20).

Animals

Male Sprague-Dawley rats (350–375 g, Jackson Laboratories, Bar Harbor, ME, USA) were divided into 3 groups ($n = 5/\text{group}$) and injected intraperitoneally (i.p. 200 μl PBS) daily for 12 days with either AGE-modified BSA (40 mg/day) (group A), AGE-modified BSA (40 mg/day) plus hen egg LZ (200 mg/day) (group B), or LZ alone (200 mg/day) (group C). Serum and 24 h-urine samples were collected at baseline and at the end of study, (each time for 3 consecutive days) for creatinine and AGE clearance (23,24). Rat or mouse serum was separated into low (LMW) (<10 kD) and high (HMW) AGE-peptide fractions as described (5). The LMW-peptide AGE values were used for calculation of AGE clearance (5).

Pre-diabetic, male, non-obese diabetic (NOD) mice (25–27 g, $n = 5/\text{group}$, Jackson Laboratories) were injected i.p., daily, for 14 days, with LZ in PBS (2 mg LZ/0.5 ml PBS/day), or PBS (0.5 ml PBS/day). In addition, ten C57BL/KsJ, db/db (+/+) mice (female, 5 month-old, Jackson Laboratories) with fasting glycemia at 390–450 mg/dL were randomly divided into two groups ($n = 5/\text{group}$). Each group received i.p. for 14 days either LZ in PBS (6 mg LZ/0.5 ml PBS/day) or PBS (0.5 ml/day). Weight-matched control SJL mice and db/m (+/-) (Jackson Laboratories) ($n = 10/\text{group}$) were injected with PBS (0.5 ml) alone. All animals were given free access to rat chow and water. At the end of the study, animals were sacrificed by decapitation, and blood, urine and kidneys were collected. Kidney cortices were snap-frozen in liquid nitrogen immediately

for total RNA, membrane protein, and total protein extraction. Kidney tissue was homogenized and digested as described (23,24), and together with aliquots of serum and urine were used for AGE determination by an AGE-ELISA (19,20). Twenty-four-hour urine creatinine levels were determined by a standard kit (Stanbio Laboratories, San Antonio, TX, USA) and albumin levels by ELISA (23,24).

Cells and Cell Assays

Macrophages Murine macrophage-like TIB-183 cells (ATCC, Rockville, MD, USA) were maintained in DMEM medium, supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) at 37°C and 5% CO₂. Cells (1 \times 10⁶ cells/well) were grown to confluency in 12-well culture plates and were then incubated in binding medium consisting of complete DMEM medium and 1% BSA for 30 min at 4°C prior to further studies. For cell binding studies, ¹²⁵I-labeled AGE-BSA, BSA or LZ (30–50 μ g/ml) were each added, in the presence or absence of 100-fold excess of unlabeled AGE-BSA, BSA, LZ, respectively, for 2 hrs at 4°C (24,25). For cellular uptake and degradation studies, similar experiments were performed using ¹²⁵I-AGE-BSA (200 μ g/ml) in the presence or absence of LZ (100 μ g/ml) at 37°C for up to 24 hrs. Also, radiolabeled AGE-enriched human sera (200 μ g/ml) were added to macrophages with or without LZ (100 μ g/ml) for 4 hrs at 37°C. Cells were washed extensively with cold PBS and lysed using 0.1 M NaOH. The radioactivity and protein content (BCA-protein analysis, Pierce) in each sample were measured. The culture media were collected, TCA-precipitated, and the radioactivity and protein content determined. All experiments were carried out in triplicate.

Mesangial Cells Glomerular mesangial cells (MC) from normal C57BL/6 mice (4 months old) were isolated and characterized, as described (24,26,27). Cell preparations were cultured in complete medium (2/3 DMEM and 1/3 F12, supplemented with 1 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal bovine serum) (Gibco) at 37°C in 5% CO₂. Cells were passaged weekly and used for no more than 8–10 passages.

Ligand Blot, mRNA and Protein Analyses

For ligand blot analysis, 10 μ g of LZ were transferred onto nitrocellulose (NC) membranes (0.2 mm pore size) using a mini-gel transfer apparatus (Bio-Rad Laboratories). The NC membranes were blocked with 2% BSA in PBS for 1 hr, washed twice with PBS containing 0.05% Tween-20 and probed with ¹²⁵I-MG-modified BSA (2 \times 10⁶ cpm) for 1 hr in the presence or absence of 50-fold excess cold AGE-BSA, BSA, CML-BSA or MG-BSA. The blots were washed extensively with PBS/Tween-20, air dried and exposed to XAR film (Kodak) at –80°C. The radioactivity associated with each blot was quantified

by a phosphorimager (Packard) and expressed as % cpm of control (uncompeted MG-BSA). This procedure was repeated at least three times.

Total RNA was extracted from cultured macrophages or mesangial cells using the phenol extraction method (RNAzol solution, TEL TEST Inc., Friendswood, TX, USA). For each extraction, RNA content was measured and equal quantities of RNA were added to reverse transcriptase reactions using a first-strand cDNA synthesis kit (Boehringer Mannheim Biochemicals Inc., Indianapolis, IN) (28,29). The primers used in PCR assays were as follows.

TNF α , sense: 5'-ACT GAA CTT CGG GGT GAT CGG TCC-3'; antisense: 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3', yielding a 293 bp product.
 IGF-I, sense: 5'-GGC TGC TTT TGT AGG CTT CAG TGG-3'; antisense: 5'-GGA CCA GAG ACC CTT TGC GGG G-3', yielding a 211 bp product.
 Tenascin, sense: 5'-TAC GAC AGA TTC AGT GTT GG-3'; antisense: 5'-TTG TAG ACT GTT GTC TTT GG-3', yielding a 548 bp product.
 α_1 type IV collagen, sense: 5'-TAG GTG TCA GCA ATT AGG-3'; antisense: 5'-TCA CTT CAA GCA TAG TGG TCC G-3', yielding a 484 bp product.
 TTA AGG TGG TGC AGG TAT CTG G-3', yielding a 701 bp product.
 MMP-2 sense: 5'-CTT TGC AGG AGA CAA GTT CTG G; antisense: 5' TAA AGG TGG TGC AGG TAT CTG G-3', yielding a 701 bp product.
 MMP-9 sense: 5'-ATG TGG TTA TGT TCA CAT GC-3'; antisense: 5'-ATG CTT CCT GGA TCA GTT CC-3', yielding a 472 bp product.
 Platelet-derived growth factor (PDGF)-B sense: 5'-TGT GAG ACA ATA GTG ACC-3'; antisense: 5'-TTG GAG TCA AGA GAA GCC-3', yielding a 432 bp product.
 TGF- β 1 sense: 5'-ATA CAG GGC TTT CGA TCC AGC-3'; antisense: 5'-GTC CAG GCT CCA AAT ATA GG-3', yielding a 360 bp product.
 β -actin sense: 5'-TCA TGA GGT AGT CCG TCA GG-3'; antisense: 5'-TCT AGG CAC CAA GGT GTG-3', yielding a 460 bp product.
 GAPDH sense: 5'-CAA AGT TGT CAT GGA TGA CC-3'; antisense: 5'-TCC TCC CTG TTC TAG AGA CAG-3', yielding a 561 bp product.

To establish the linear range of PCR amplification for the above molecules, we plotted increasing numbers of PCR cycles against the integrated optical density obtained from computer-aided densitometry. The optimized PCR cycles were 30 for TNF; 30 for IGF-I; 24 for Tenascin; 23 for α_1 type IV collagen; 32 for MMP-2; 34 for MMP-9; 30 for PDGF-B; 30 for TGF- β 1; 24 for β -actin and 20 for GAPDH. There were no changes in the "housekeeping" genes, β -actin and GAPDH expression levels in cells treated with or without AGE (data not shown). Since the levels of β -actin and GAPDH mRNA were

comparable in each of our cDNA samples, PCR data was normalized to β -actin as a control of interassay variance (28,29). At least 5 RT-PCRs were conducted for each primer pair using cells from separate preparations.

Tumor Necrosis Factor (TNF)- α and Insulin-like Growth Factor-I (IGF-I) Protein Assessment

Cells from the mouse macrophage cell line TIB-183 (ATCC, Rockville, MD, USA) were plated at 10^6 cells/well into 6-well plates with RPMI 1640 and 10% FCS medium at 37°C and 5% CO₂. After 24 h, cells were washed three times with PBS and incubated with AGE-modified BSA, BSA (100 μ g/ml), or MG-BSA (25 μ g/ml), in the presence or absence of 100 μ g/ml LZ for 24 hrs. Rabbit anti-hen egg LZ antibody (50 μ g/ml) was added in a separate series together with LZ (100 μ g/ml) to test the specificity of the LZ effects. Pre-immune rabbit IgG was used as control. Total RNA was extracted from cells 24 hrs after treatment, and reverse-transcribed with random primers, as described above (24,28,29). Secreted TNF α and IGF-I were determined in the medium at 48 hrs, using a mouse TNF α immunoassay kit (Biosource International, Camarillo, CA, USA) and a rat IGF-I immunoassay kit (Diagnostic System Laboratory Inc, Webster, TX, USA), respectively.

PDGF-B, α_1 Type IV Collagen, Tenascin, and MMP-2 and MMP-9 in Mesangial Cell

Mesangial cells (MC) were plated in 6-well plates at a density of 1×10^5 cells/well in DMEM/F12 + 20% fetal calf serum (FCS) as previously described (24,26). Twenty-four h later, the cells were washed twice with PBS and the culture medium was replaced with DMEM/F12 + 0/5% FCS. AGE-BSA (800 μ g/ml) or native BSA (800 μ g/ml) was added with or without hen egg LZ (400 μ g/ml). Cells were collected after 72 hours of incubation. Cellular RNA extraction and quantitative RT-PCR analysis was performed as described (26,29), using the primers described above. β -actin and GAPDH were used as housekeeping genes. Because both genes provided identical results, only β -actin data are shown.

MMP Activity

Mesangial cell MMP-2 and MMP-9 expression and activity were assessed (30,31). MC (1×10^6 /well) were incubated with DMEM/F12 + 0.1% BSA in the presence of AGE-BSA or BSA (300 μ g/ml) with or without lysozyme (150 μ g/ml). After 24 h, the cell culture medium was collected and loaded onto a 10% gelatin gel (BioRad). The gel was then incubated with 2.5% Triton-X at room temperature for 1 h, and then placed in collagenase buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.2% Brij-35) at 37°C for 24 h. Gelatinase activity was visualized by staining the gel with 0.5% Coomassie Blue, and subsequent destaining with 30% methanol and 10% acetic acid.

Statistics

Analysis of variance and Student's *t* test (unpaired, two-tailed) were used to analyze differences between groups. P values <0.05 were considered statistically significant.

Results

LZ Reduces Endogenously-formed Serum AGE and Increases Renal AGE Excretion

To test the ability of LZ to lower *in-vivo* formed sAGE, we studied non-obese-diabetic (NOD) mice. While less susceptible to diabetes, male NOD are more susceptible to nephropathy and importantly they exhibit elevated serum AGE levels (24), which provided the advantage of examining LZ effects on elevated native AGE *in vivo*, but in the absence of hyperglycemia, studied below in the *db/db* mouse strain. As expected (24), NOD mice exhibited higher sAGE levels than control mice (31.5 ± 6.7 vs 14.2 ± 5 U/ml, $p < 0.05$). The administration of LZ for 14 days decreased NOD serum AGE levels to 22.3 ± 5 U/ml, $p < 0.05$, an effect accompanied by a significant and proportional increase in urinary AGE excretion by 1.8-fold at 2 weeks (from 200 ± 71.4 U/ml to 367.9 ± 41.2 U/ml, $p < 0.05$). These effects occurred without significant changes in creatinine clearance, nor in fasting blood glucose or body weight (Table 1). No effect in sAGE nor in uAGE was seen after administration of PBS as control (not shown).

The effect of 2-week LZ administration was also tested in a spontaneously diabetic *db/db* (+/+) mouse model, exhibiting type II diabetes, obesity and insulin resistance. LZ treatment reduced the elevated baseline sAGE levels in these mice (from 37.9 ± 3.8 to 26.2 ± 5.3 U/ml, $p < 0.05$, control: 21.5 ± 7.7 U/ml), and nearly doubled the urinary excretion of AGE (from 92.7 ± 52.6 to 170.9 ± 49.5 U/ml, $p < 0.05$), made more evident when expressed as AGE clearance ($p < 0.01$) (Table 1). These effects were not associated with changes in creatinine clearance, fasting blood glucose (320 ± 130 mg/dL before vs. 290 ± 60 mg/dL after), or body weight. No effects on sAGE or uAGE clearance were observed in NOD mice injected with PBS alone (data not shown).

Baseline urinary albumin (UA) levels in both NOD and *db/db* mice were moderately increased over those of normal controls. Over the two-week period during which NOD mice were injected with LZ, UA declined by 35% ($p < 0.005$). Similarly, LZ treatment of *db/db* mice reduced UA excretion by 65% ($p < 0.01$) (Table 1).

The time-dependent effects of LZ administration were tested on normal rats infused with exogenous AGE-BSA. While in rats administered AGE alone (Group A), serum AGE (sAGE) levels rose significantly and reached a 2.2-fold increase above baseline within 12 days ($p < 0.01$), in rats administered both AGE and LZ (Group B), sAGE levels remained near baseline despite continuing AGE infusion for 12 days

Table 1. Effect of a 2-week course of LZ administration on physiological serum and urine characteristics of NOD and db/db mice

| Treatment (LZ) | SJL (n = 10) | NOD (n = 5) | | db/m (n = 10) | db/db (n = 5) | |
|---------------------------|-----------------|----------------|------------------|------------------|------------------|-----------------|
| | | Baseline | 2-Weeks | | Baseline | 2-Weeks |
| Weight (g) | 25 ± 3 | 25.3 ± 2.4 | 26.8 ± 1.6 | 26.7 ± 2.8 | 48.9 ± 1.5 | 50.1 ± 1.6 |
| Blood glucose (mg/dL) | 117 ± 44 | 128 ± 20 | 132 ± 32 | 121 ± 27 | 320 ± 130 | 290 ± 60 |
| Serum AGE (U/ml) | 14.2 ± 4.8 | 31.5 ± 6.7 | 22.3 ± 5.1* | 21.5 ± 7.7 | 37.9 ± 3.8 | 26.2 ± 5.3* |
| Serum Cr (mg/dL) | 0.38 ± 0.04 | 0.42 ± 0.02 | 0.47 ± 0.05 | 0.33 ± 0.06 | 0.69 ± .01# | 0.72 ± 0.11## |
| Urine AGE (U/ml) | 197 ± 41 | 200.3 ± 71.4 | 367.9 ± 41.2* | 103.8 ± 37.5 | 92.7 ± 52.6 | 170.9 ± 49.5* |
| Urine Cr (ug/ml) | 357.3 ± 77.9 | 308.4 ± 68.7 | 324.9 ± 50.3 | 276.5 ± 93.4 | 207.6 ± 31.7 | 212.3 ± 49.4 |
| Ccr (ml/min) | 0.03 ± 0.01 | 0.04 ± 0.008 | 0.05 ± 0.007 | 0.05 ± 0.02 | 0.05 ± 0.007 | 0.05 ± 0.01 |
| C _{AGE} (ml/min) | 0.004 ± 0.0012 | 0.003 ± 0.0011 | 0.008 ± 0.0009** | 0.003 ± 0.001 | 0.004 ± 0.002 | 0.013 ± 0.004** |
| Urinary Alb/Cr | 0.29 ± 0.15 | 0.92 ± 0.32## | 0.53 ± 0.24* | 0.38 ± 0.2 | 1.13 ± 0.45 | 0.73 ± 0.26* |

*p < 0.05, **p < 0.01, 2 weeks LZ vs baseline. #p < 0.05, ##p < 0.01, vs respective control group.

(Fig. 1A). In addition, in the LZ + AGE group (group B), LZ administration was associated with a time-dependent increase in urinary AGE (uAGE) clearance (max. 2.1-fold) above the AGE alone group (Group A), and in proportion to the reduction in sAGE (p < 0.01), although renal creatinine clearance remained stable ($98 \pm 12 \times 10^{-5} \text{ ml} \cdot \text{min}^{-1}$) throughout the experiment (Fig. 1B). Of note, control animals that were injected with LZ also exhibited a significant increase in endogenous uAGE excretion (50% of untreated controls, p < 0.01), which was associated with a similar decrease in sAGE levels, indicating a salutary LZ effect on normally excretable AGE.

Lysozyme Increases the Macrophage Binding, Endocytosis and Degradation of AGE

In vivo studies (Table 1) and (Fig. 1) demonstrate that, full-length LZ-polypeptide administered to either spontaneously diabetic mouse models or non-diabetic AGE-infused rats, reduces super-normal

levels of circulating AGE (sAGE) within a period of 2 weeks. While this effect is attributable, at least in part, to increased renal clearance of AGE, other indirect mechanisms might be involved, including cellular sequestration, and processing of AGE bound by LZ before their release from tissues into the circulation and ultimately in the urine. To examine the cell-related uptake of AGE in the presence of LZ, we examined two relevant cell types, macrophages and primary mouse mesangial cells, both known to interact with AGE (9,24,26,28).

Macrophage surface-binding of ^{125}I -AGE-BSA exhibited the expected AGE-specific pattern, based on its effective inhibition by added AGE ligand. In the presence of LZ, AGE-specific binding increased significantly (Fig. 2A). In contrast, ^{125}I -LZ binding by these cells when added alone was not significant, nor did it change in the presence of additional LZ, a result similar to that obtained using native ^{125}I -BSA as control, indicating that LZ interacted only minimally

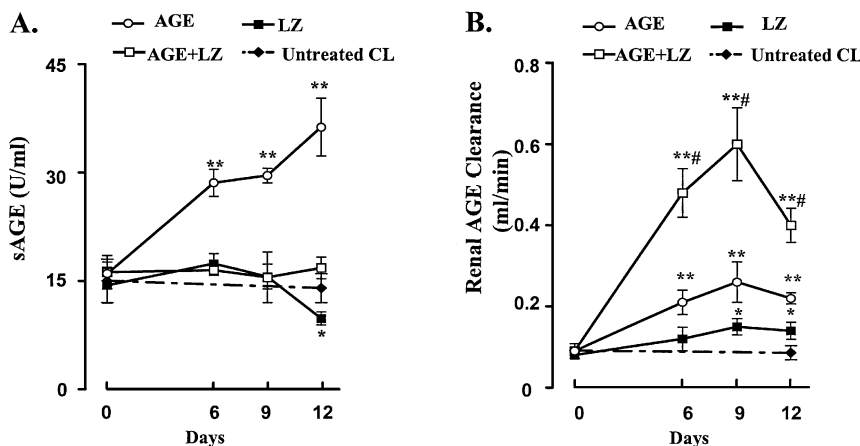


Fig. 1. Lysozyme reduces serum AGE (A) and enhances renal AGE-clearance (B) in AGE-treated, non-diabetic rats. AGE-BSA (40 mg), AGE-BSA (40 mg) + hen egg LZ (200 mg), LZ (200 mg) or PBS were injected daily i.p. into normal rats (n = 5/group) for 12 days. Serum AGE (sAGE) levels are expressed as U/ml and urinary AGE clearance as ml/min. Each point represents the mean \pm standard deviation ($M \pm SD$) of triplicate values obtained from each animal group. **p < 0.01 vs control, *p < 0.05 vs. control, #p < 0.01 vs AGE + LZ group.

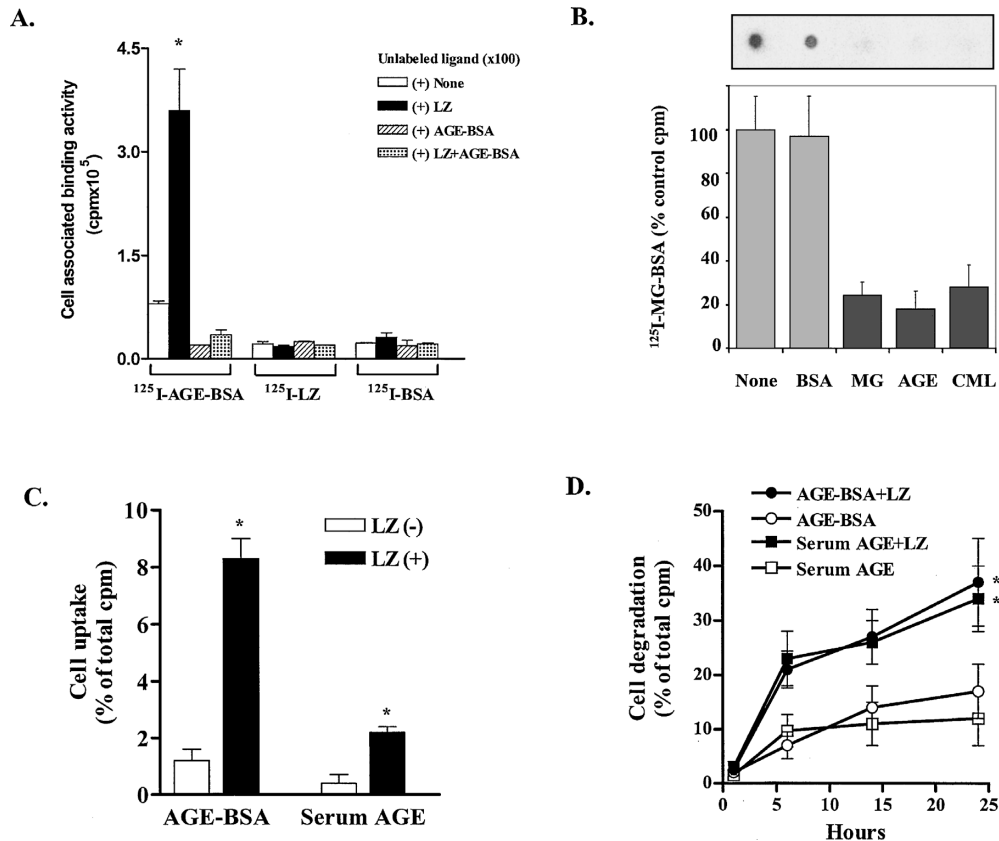


Fig. 2. Lysozyme enhances macrophage binding, internalization and degradation of AGE formed *in vitro* or in human sera. (A) Confluent cultures of murine macrophage-like T1B-183 cells (0.5×10^6 /well) were incubated with either ^{125}I -AGE-BSA, ^{125}I -LZ or ^{125}I -BSA ($10\text{--}25 \mu\text{g}/\text{well}$), in the presence or absence of 100-fold excess of the indicated unlabeled ligand, for 2 hrs at 4°C . (B) LZ was immobilized on a NC membrane ($10 \mu\text{g}/\text{dot}$) and probed with ^{125}I -MG-BSA (4×10^6 cpm, or $5 \mu\text{g}/\text{ml}$) in the presence or absence of 50-fold excess of unlabeled AGE-BSA, MG-BSA, CML-BSA or BSA for 1 hr. Radiolabeled ligand binding was visualized by exposure to XAR film (Kodak) at -80°C (upper), and quantitated by phosphorimage analysis of three identical ligand dot blots, expressed as $M \pm \text{SD}$ (lower). (C) T1B-183 cells, plated as in (A) were incubated with either ^{125}I -labeled AGE-BSA ($100 \mu\text{g}/\text{well}$) or ^{125}I -human AGE-enriched serum ($150 \mu\text{g}/\text{well}$) with or without LZ ($80 \mu\text{g}/\text{ml}$) for 4 hrs at 37°C . (D) Media from T1B-183 cell cultures treated as in (C) were collected and after TCA-precipitation, soluble cpm were determined. For (A) (C) and (D) panels, data are expressed as the $M \pm \text{SD}$ cpm of five independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ vs. LZ.

with the cell surface and in a non-specific manner. In a separate study, LZ immobilized onto a NC membrane was incubated with ^{125}I -MG-modified BSA (1×10^6 cpm/ml) in the presence or absence of 50-fold excess unlabelled AGE-modified BSA, MG, CML or native BSA. As shown in Fig. 2B, ^{125}I -MG-BSA bound onto LZ in a manner that was competitively inhibited by excess MG, AGE-BSA, or CML, but not by unmodified BSA, confirming that AGE recognition and binding by LZ included specific glycoxidative species found *in vivo*, such as MG or CML.

Furthermore, endocytosis and degradation of ^{125}I -AGE-BSA or ^{125}I -human serum AGE by macrophages were also enhanced in the presence of LZ (at 37°C) (Fig. 2, C–D).

LZ Suppresses AGE-mediated Macrophage Activation

Since AGE also induce gene activation in several cellular systems (3,9), we examined the effect of LZ on select AGE-dependent responses. The AGE-induced elevation of $\text{TNF}\alpha$ mRNA levels was abrogated in

the presence of LZ (Fig. 3, A–B). Also, the AGE-stimulated expression of IGF-I mRNA was inhibited in the presence of LZ (Fig. 3, E–F). Of note, the effects of LZ were reversed by the addition of anti-LZ antibody. Furthermore, when added to cells, the MG-enhanced $\text{TNF}\alpha$ mRNA expression was also abrogated in the presence of LZ (Fig. 3, C–D). Also, LZ blocked the enhancing effect of AGE on macrophage $\text{TNF}\alpha$ and IGF-I protein secretion (Fig. 4, A–B).

LZ Suppresses AGE-mediated Mesangial Cell Activation

As noted previously (3,26), normal mouse mesangial cells after incubation with AGE exhibited enhanced expression of PDGF-B, α_1 type IV collagen and tenascin mRNA; however, in the presence of LZ, this response was inhibited (Fig. 5, A–C). LZ alone had no effect in any of these studies (Table 2).

Extracellular matrix is under the regulatory control of an important family of proteolytic enzymes, the metalloproteinases (MMP), including MMP-2 and MMP-9, believed to be important to the kidney. AGE

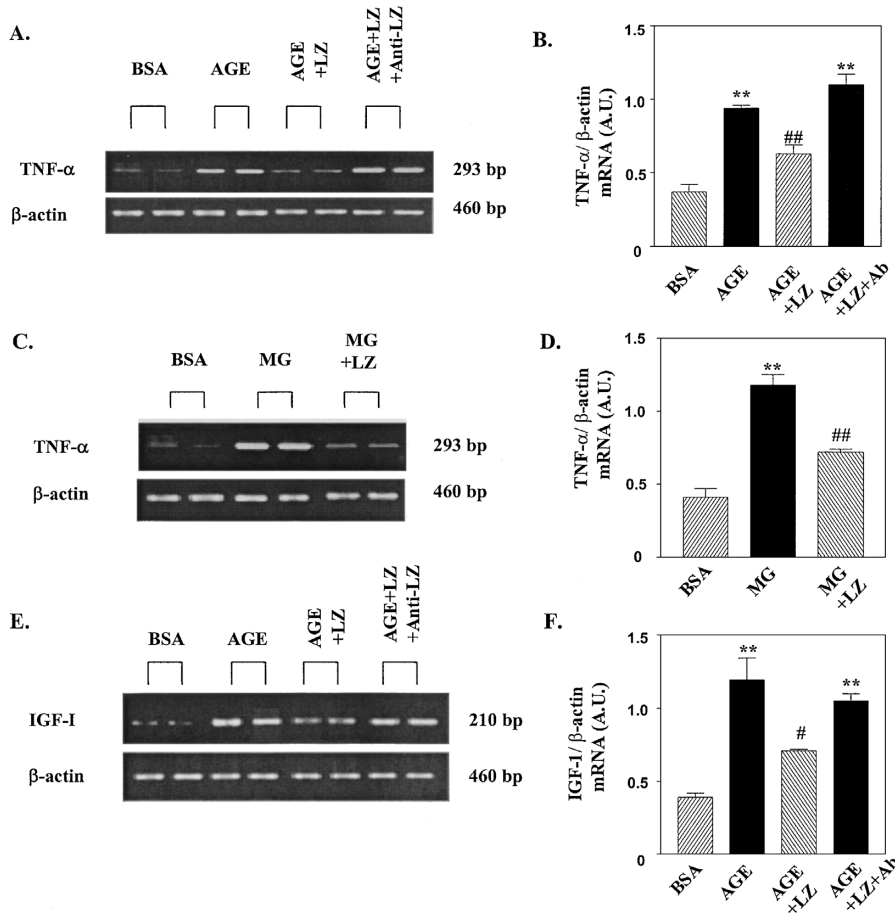


Fig. 3. Lysozyme suppresses AGE-mediated macrophage gene induction. Confluent cultures of murine macrophage-like T1B-183 cells (1×10^6 /well) were incubated with either AGE-BSA (200 μ g/ml) or BSA (200 μ g/ml), in the presence or absence of LZ (100 μ g/ml), which was added with or without anti-LZ antiserum (50 μ g/ml) for up to 24 hrs at 37°C. Total RNA was extracted for TNF α (A,B), and IGF-I (E,F) mRNA assessment by RT-PCR. (C,D) MG-BSA (25 μ g/ml) was added to T1B-183 cells with/without LZ (100 μ g/ml) and RNA was extracted for TNF α mRNA testing by RT-PCR. β -actin is shown as an internal control gene. Data are representative of at least three identical experiments, each done in duplicate.

exerted a markedly suppressive effect on MMP-9 gene expression, estimated to be ~55% below control, based on RT-PCR and densitometric analysis (Fig. 5, A–B), but no effect of AGE was seen on MMP-2 mRNA (data not shown). AGE also suppressed MMP-9 proteolytic activity against gelatin, but not of MMP-2 based on zymography (30,31) (Fig. 5, C–E). In the presence of LZ, however, AGE-mediated suppression of MMP-9 mRNA expression and proteolytic activity were ameliorated (Fig. 5, C–D).

Discussion

Pharmacological interventions aimed at bioactive AGE have been effective against the complications of

diabetes and aging (11,12,23). We have recently found that the well-known anti-bacterial protein lysozyme (LZ) contains an AGE-specific-binding, cysteine-bounded domain, (ABCD motif), by which it can engage and sequester AGE-modified substances from diabetic serum (17,18). In the present study, we explored the *in-vivo* effects of native (full-length) LZ polypeptide, employed as a soluble AGE receptor on the sequestration and removal of circulating AGE, using two murine diabetes models, with elevated endogenous, circulating AGE levels, and a non-diabetic rat model exogenously supplemented with AGE. Synthetically produced AGE-binding LZ-peptide (ABCD) has been found equally active *in vitro*

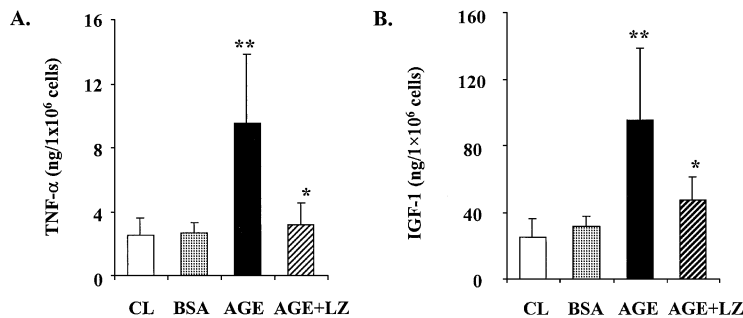


Fig. 4. Lysozyme suppresses AGE-mediated synthesis of TNF α and IGF-I by macrophages. Macrophage-like T1B-183 cells (1×10^6 /well) were incubated with either AGE-BSA (200 μ g/ml), BSA (200 μ g/ml), or serum-free medium (CL) in the presence or absence of LZ (100 μ g/ml), for 48 hrs at 37°C. Conditioned media were tested for TNF α (A) and IGF-I (B) content by immunoassay. Data are expressed as the $M \pm SD$ ng/well (1×10^6 cells) of three identical experiments, each done in duplicate. * $p < 0.01$ vs. AGE, ** $p < 0.001$ vs. control.

Table 2. LZ suppresses AGE-induced mesangial cell expression of growth promoting genes in culture⁵

| mRNA* | Control | LZ | AGE | AGE + LZ |
|--|-----------------|------------------|-------------------|-------------------|
| PDGF-B/ β -actin(A.U.) | 0.12 \pm 0.05 | 0.164 \pm 0.04 | 0.49 \pm 0.11** | 0.27 \pm 0.07# |
| TGF- β 1/ β -actin (A.U.) | 0.21 \pm 0.04 | 0.19 \pm 0.08 | 0.24 \pm 0.09 | 0.26 \pm 0.03 |
| α 1 (IV)/ β -actin (A.U.) | 0.27 \pm 0.07 | 0.29 \pm 0.13 | 0.74 \pm 0.12** | 0.39 \pm 0.06# |
| Tenascin/ β -actin (A.U.) | 0.05 \pm 0.01 | 0.04 \pm 0.02 | 0.19 \pm 0.06** | 0.07 \pm 0.02## |

**p < 0.05, vs Control. #p < 0.05, ##p < 0.01, vs AGE.

*Data are expressed as the ratio of each factor cDNA/mutant to β -actin cDNA/mutant and are shown as the M \pm SD of three experiments, each performed in duplicate.

⁵Primary murine mesangial cell cultures (1×10^5 /well) were incubated with AGE-BSA (800 μ g/ml), AGE-BSA (800 μ g/ml) + LZ (400 μ g/ml) or media (DMEM/F12 + 20% FCS) (Control) or LZ alone (400 μ g/ml) for up to 3 d. mRNA expression for PDGF-B, α 1 type IV collagen, or tenascin was determined by RT-PCR.

(17,18), indicating that the physiological effects observed are due to AGE-binding of LZ. To avoid its prompt renal elimination, however, we chose to use the full-length lysozyme molecule in these studies.

In support of the potential clinical utility of LZ were the reduction of diabetic level sAGE and the enhanced clearance of urinary AGE. These effects were similar in two models of spontaneous diabetes, type 1 (NOD) and type 2 (*db/db*), both of which are subject to developing diabetic nephropathy (31,32). These effects also indicated that LZ, if administered

in vivo, will maintain its AGE-binding activity even under diabetic conditions.

In a parallel study, pre-loading of normal rats with *in vitro* made AGE, followed by LZ treatment also led to a reduction of serum AGEs, and a proportional increase in urinary AGE levels. This supported the hypothesis that LZ can function in circulation as a carrier molecule for pre-formed AGE, such as those that are diet derived, and can speed their removal by the kidney. The same study also revealed a significant reduction in rat serum sAGE

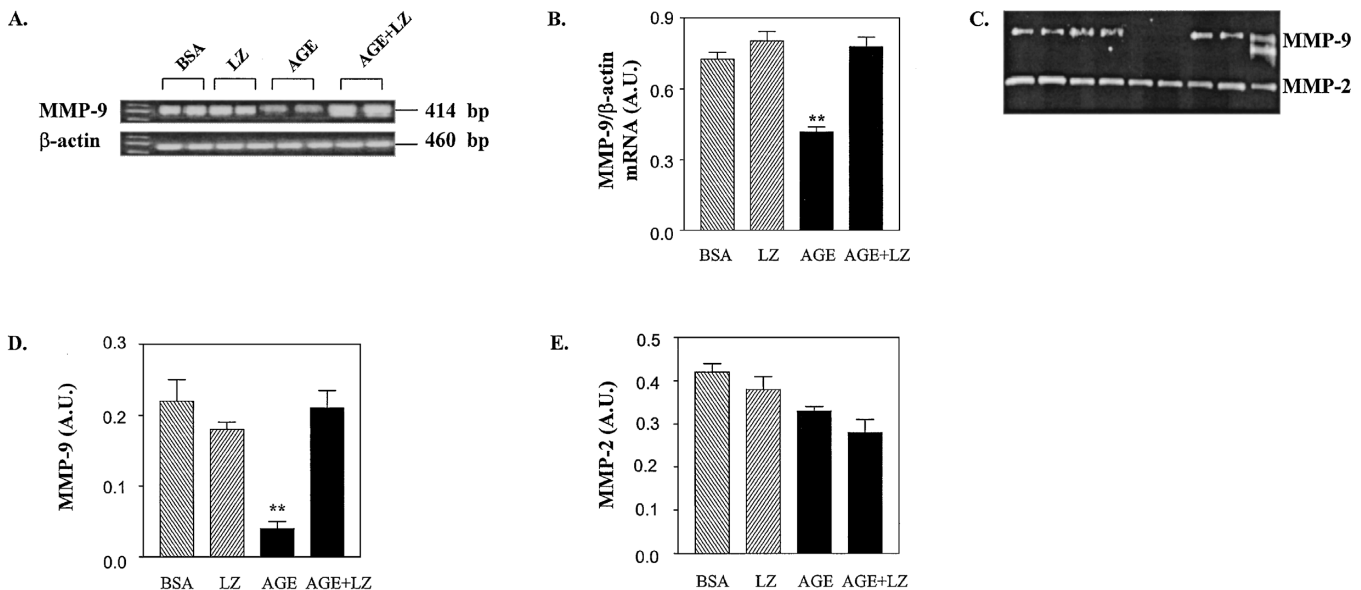


Fig. 5. LZ prevents AGE-suppressible gene expression, and proteolytic activity of mesangial cell MMP-9, but not MMP-2.

(A) Primary murine mesangial cell cultures (1×10^6 /well) were incubated with AGE-BSA, BSA (500 μ g/ml each), AGE-BSA + LZ (200 μ g/ml) or media (DMEM/ F12 + 20% FCS) (CL) for 24 hr at 37°C. RT-PCR for MMP-2 and MMP-9 was performed as described in Methods, β -actin is shown as an internal control gene. (B) Densitometric analysis of triplicate experiments represented in panel (A). ** p < 0.001 vs AGE + LZ. (C) MMP-9 and MMP-2 activity in mesangial cell extracts was assessed by zymography, based on gelatinase activity, gels were stained with Coomassie Blue (0.5%). From left to right, lanes identities are the same as in (A), in pairs, with the addition of standard (MMP-9, -2). (D,E) Densitometric analysis of three identical experiments on MMP-9 and MMP-2 activities as presented in panel (C). **p < 0.001 vs AGE + LZ.

well below the "normal" range, further confirming that LZ binds equally well to endogenous normally produced AGE.

Relatively little is known about the precise mechanism of renal handling of AGE beyond the fact that AGE clearance correlates with creatinine clearance or glomerular filtration rate (5) and that both the glomerulus and the proximal tubule are involved in AGE turnover and clearance (33,34). LZ has been shown to be readily filtered through the glomerulus and to be taken up in part by the proximal tubule, for proteolytic digestion, mainly by cathepsin-D (35,36) before being excreted into the urine. Thus, the effects of LZ on renal AGE clearance could be explained in part by enhanced filtration of LZ-captured AGE via the glomerulus. There may be a partial AGE re-uptake by the proximal tubule, where the AGE bound to LZ is degraded and AGE-LZ-peptides are excreted rapidly into the urine. This however remains to be further elucidated.

Interestingly, LZ treatment, however brief, exerted an effect on the progression of albuminuria that was already present in both diabetic mouse models. The LZ effect, exerted against modest degrees of albuminuria in this study, although moderate, was nonetheless suggestive of a potentially novel property of LZ: an ability to effectively abort certain interactions between AGE and renal cells, and/or basement membrane, that would otherwise result in perturbations of permeability and/or selectivity for albumin. This effect was similar in two animal models despite the different types of diabetes and metabolic states. This focuses the action of LZ on glycoxidation derivatives, while keeping them distinct from glucose or insulin actions. While LZ activity could involve the prevention of intracellular reactive oxygen species (ROS) generated by AGE (3, 10,37), this intriguing hypothesis is under investigation. No obvious metabolic nor renal functional parameters, e.g., glycaemia, insulinaemia, creatinine clearance, were influenced by the 2-week-long LZ treatment; the chronic effects of LZ remain to be shown, pending the availability of recombinant autologous LZ peptides.

Because of the immunological constraints related to the longer-term *in vivo* use of a heterologous LZ polypeptide, and in order to expand on the mechanisms of LZ action, our attention turned to relevant cellular systems, namely matrix-producing mesangial cells (MC) and AGE-scavenging macrophages (9,38,39). Both processes appeared enhanced in the presence of LZ, pointing to a potential tool for speedy AGE removal *in vivo*.

The possibility existed, however, that the LZ-enhanced AGE uptake might give rise to a more intense AGE-inflammatory response (9,40). On the contrary, LZ effectively and consistently blocked AGE-induced expression of several known inflammatory genes, such as TNF α and IGF-I (39,40). This same effect was also observed when a single, defined

glycoxidation species derived from MG and albumin (MG-BSA) was studied. Derivatives of MG have been associated with immune cell activation and oxidant stress induction (41,42). The neutralizing effect of LZ on MG-BSA shown herein strongly supports the specificity of LZ for *in-vivo* pathogenic AGE derivatives (7,8,21,41) and furthermore it suggests a suppressive effect against cellular oxidative stress.

Additional findings from studies on the developmentally distinct and kidney-specific mesangial cell provided further evidence relevant to the *in-vivo* utility of LZ, e.g., against diabetic kidney dysfunction. A consistent pattern of suppression of several MC growth and matrix regulatory genes, triggered by AGE (26,43) was exhibited by LZ. A novel finding of note was the reversal by LZ of the AGE-specific suppression of expression and activity of MMP-9. This molecule is an important member of the renal matrix degrading system, and is suppressed in glomerulosclerosis (30,31); no effect was seen on MMP-2, however, by either AGE or LZ. Together, these data indicate that cellular functions, specifically targeted by AGE may include those most responsive to treatment by LZ. The role of LZ in effectively inhibiting mesangial proliferation in the intact diabetic kidney remains an important subject for future studies. On the mechanistic level, it is conceivable that LZ engages reactive carbonyl-bearing AGE within its hydrophilic ABCD loop, thereby concealing or stabilizing reactive moieties that bear the potential for ROS formation or release (3,10,37). By its cationic nature, the LZ molecule can also form larger complexes with negatively charged AGE-peptides, which are taken up avidly by macrophages. As ROS-generating moieties are rendered inaccessible by LZ, AGE may be processed through the lysosomal system without provoking pro-inflammatory signals. This view may bear a relationship to the neutralizing action of LZ on potent cellular activators of oxidative stress, such as noted previously with bacterial LPS (44) or viral stimuli (45). The specific molecular pathways remain to be determined; however, preliminary evidence using cultured macrophages over-expressing recombinant LZ, either full-length or as peptides lacking specific domains, e.g., signal peptide or the ABCD domain, indicated that in the absence of the ABCD peptide, the endocytosis-enhancing and cytokine-blocking properties of LZ were ameliorated (46). This may serve as a direct link between the AGE-binding, and the biological effects of LZ reported herein. Furthermore, evidence indicating enhanced LZ expression in mouse diabetic kidney may be consistent with a natural anti-glycoxidative role for this molecule.

The protection noted against local diabetic tissue damage, e.g., atheroma formation, by another AGE-binding peptide, sRAGE was promising (47), however, the ultimate disposal of the RAGE: AGE complex was not addressed in these studies. In the

present report, the combined evidence of anti-inflammatory action in cultured cells and enhanced *in vivo* AGE elimination provides a reasonable basis for the assertion that LZ is not likely to result in AGE deposition elsewhere nor with "delayed" secondary cellular activation and toxicity.

In conclusion, the host-defense protein lysozyme possesses a unique combination of anti-AGE properties: accelerated cellular AGE turnover, neutralization of pro-inflammatory signals *in vitro*, and enhanced renal AGE elimination *in vivo*. These advantages may prove instructive in the design of novel therapies against diabetic complications.

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