Vascular Biology, Atherosclerosis and Endothelium Biology

Reduced Acute Vascular Injury and Atherosclerosis in Hyperlipidemic Mice Transgenic for Lysozyme

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Hyperlipidemia promotes oxidant stress, inflammation, and atherogenesis in apolipoprotein E-deficient (ApoE^(-/-)) mice. Mice transgenic for lysozyme (LZ-Tg) are resistant to acute and chronic oxidative stress and have decreased circulating levels of pro-oxidant advanced glycation end-products (AGEs). Herein we report that TIB-186 macrophages transduced with adenovirus-expressing human LZ (AdV-LZ) containing the AGE-binding domain facilitated AGE uptake and degradation and that AdV-LZ-transduced macrophages and peritoneal macrophages from LZ-Tg mice suppressed the AGE-triggered tumor necrosis factor- α response. We assessed atherosclerosis in LZ-Tg mice crossed with ApoE^(-/-) mice (LZ/ApoE^(-/-)) and found increased serum LZ levels and decreased AGE and 8-isoprostanes levels, although hyperlipidemia remained similar to ApoE^(-/-) controls. Atherosclerotic plaques and neointimal lesions at the aortic root and descending aorta were markedly decreased (by 40% and 80%, respectively) in LZ/ApoE^(-/-) versus ApoE^(-/-) mice, as were inflammatory infiltrates. The arterial lesions following femoral artery injury in LZ/ApoE^(-/-) mice were suppressed (intimal to media ratio decreased by 50%), as were AGE deposits and vascular smooth muscle cell activation, compared to ApoE^(-/-) mice. Despite hyperlipidemia, development of atheroma and occlusive, inflammatory arterial neointimal lesions in response to injury was suppressed in LZ/ApoE^(-/-) mice. This effect may be due to the antioxidant properties of LZ, which is possibly linked to the AGE-binding domain region of the molecule. (*Am J Pathol* 2006, 169:303–313; DOI: 10.2353/ajpath.2006.050885)

Cardiovascular disease, the leading cause of morbidity and mortality throughout the world, is increased by risk factors such as diabetes mellitus, hyperlipidemia, hypertension, and aging.^{1–5} Oxidant stress due to excess formation of reactive oxygen species (ROS) is recognized as a major source of cell injury and inflammatory response, underlying vascular and metabolic disorders.^{6–9} Under normal conditions, where ROS play a part in normal metabolism and antioxidant defenses are intact, the body is able to successfully manage nonpathological amounts of exogenous or endogenous oxidants and avoid oxidant stress.^{9,10} Increasing evidence, however, points to excess oxidant stress, causing a negative shift in redox balance and resulting in multiple abnormalities in gene responses.^{11–13} The sources of excess levels of oxidants leading to stress are multiple, especially in metabolic disorders such as in diabetes and dyslipidemia.^{14,15} A significant part of the oxidant load *in vivo* is due to advanced glycation end-products (AGEs) from proteins or advanced lipoxidation end-products.^{14,15} These products are ubiquitous and abundant within cells and in the extracellular milieu, constituting important contributors to ROS production and oxidant stress, redox-sensitive transcription activity, and inappropriate inflammatory responses in disease.^{7,9,11,16} It has been shown that the diet can be an important exogenous source of excess oxidants.^{17–22}

The promotion of elevated oxidative stress by an inflammatory response, or by AGEs, has been demonstrated *in vitro* and in animal studies and confirmed in patients with diabetes mellitus and/or end-stage renal disease.^{15,20–26} Decreasing the levels of AGEs, by either drugs or low-AGE-containing diets, results in amelioration of cardiovascular or kidney lesions in animals^{23–33} and diminishes the level of inflammatory mediators in the serum of patients with diabetes or renal disease.^{34,35} These observations led to the concept that chronic oxidative stress, induced by environmental agents such as

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AGEs, may play an important role in the genesis of cardiovascular disease.²⁴

Another mechanism to reduce acute and chronic injury due to oxidants such as AGEs is by the administration of lysozyme, a naturally occurring immune defense protein.^{36,37} Lysozyme is present in high concentration in various tissues and fluids, including liver, articular cartilage, saliva, milk, and tears. It is highly expressed in granulocytes, monocytes, and macrophages as well as in the bone marrow precursors. Lysozyme is a well-characterized bacteriolytic enzyme that preferentially hydrolyzes the β -1,4-glycosidic linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine that occur in the peptidoglycan bacterial wall structure. It is unlikely that lysozyme has a direct interaction with lipopolysaccharide (LPS), an important component of gram-negative bacteria. The fact that lysozyme is highly active against many gram-positive species, but ineffective against gram-negative bacteria, supports this point of view. We have reported that a well-conserved 18-amino acid cysteine-bounded domain (ABCD) in lysozyme is a high affinity AGE-binding domain.^{38,39} AGEs bind to lysozyme (LZ) in human serum,³⁸ rendering LZ less functional.³⁹ Thus, the levels of LZ may be in the normal range, even though their antioxidant properties are impaired. When administered *in vivo*, LZ is associated with increased AGE clearance and suppressed AGE-induced secretion of tumor necrosis factor- α (TNF- α), platelet-derived growth factor B, insulin-like growth factor-1, α 1 type IV collagen, and tenascin, as well as matrix metalloproteinase 9 in diabetic mice.⁴⁰ We recently found that LZ administration suppressed ROS generation and oxidant stress response gene transcription and provided protection against acute and chronic oxidant injury.³⁷

In the current study we first asked if overexpression of ABCD-containing LZ peptides by macrophages would mimic those of exogenous LZ administration, eg, enhance AGE removal and suppress AGE-induced oxidative stress. Secondly, using LZ/apolipoprotein E-deficient (ApoE^{-/-}) mice, crosses between ApoE^{-/-} mice, which develop complex atherosclerotic lesions that resemble human lesions,^{23,26} and mice transgenic for hen-egg lysozyme (LZ-Tg),⁴¹ we evaluated the *in vivo* effect of endogenously overexpressed LZ on markers of oxidant burden, atheroma formation, and neointimal formation after injury.

Materials and Methods

Reagents

Human lysozyme (hLZ), cell culture-tested fatty acid- and endotoxin-free bovine serum albumin (BSA, fraction V, Sigma), and LPS from *Escherichia coli* serotype 026:B6 were purchased from Sigma-Aldrich (St. Louis, MO). Sodium ¹²⁵I-iodide was obtained from DuPont-Merck Pharmaceutical Co. (Wilmington, DE). Polyclonal sheep anti-hLZ was purchased from Alpco, Windham, NH, and anti-hen-egg lysozyme was from Accurate Chemical Scientific Corp., Westbury, NY. Monoclonal mouse anti-

green fluorescence protein (GFP) antibody was purchased from Qbiogene, Inc. (Montreal, QC, Canada). TNF- α and insulin-like growth factor-1 mouse immunoassay kits were from Biosource International (Camarillo, CA). Anti-CD68 and α -smooth muscle cell antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation and Iodination of AGEs

AGE-modified BSA (AGE-BSA) was prepared as described previously.^{37,38} Briefly, BSA was incubated with or without 0.5 mol/L D-glucose in 0.2 mol/L phosphate buffer (pH 7.4) at 37°C for 6 weeks under sterile conditions; low molecular weight reactants and glucose were removed by extensive dialysis. AGE levels on AGE-BSA were 250 U/mg protein or on unmodified BSA were 0.9 U/mg based on a competitive AGE-enzyme-linked immunosorbent assay (ELISA).⁴² Endotoxin was removed using an endotoxin-binding affinity column (Pierce, Rockford, IL). BSA and AGE-BSA contained <0.2 ng/ml endotoxin (E Toxate, Sigma-Aldrich). Aliquots of each protein preparation were iodinated using the Iodo-Beads method (Pierce), as previously described.⁴⁰ Using 20% trichloroacetic acid precipitation, it was determined that >95% of the resulting ¹²⁵I-counts per minute (cpm) was protein-associated (specific activity: ~ 0.8 to 1.0×10^3 cpm/ng).

Recombinant Adenoviral Vectors

A full-length human lysozyme (hLZ) cDNA open reading frame containing the signal peptide was amplified by the polymerase chain reaction (PCR) from human lung cDNA library (Clontech, Palo Alto, CA) and specific primers for human lysozyme, according to the published sequence of the gene (NCBI sequence accession number NM_000239). Amplified PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Two hLZ mutants, one lacking the signal peptide 1–18 (AdV-hLZ Δ 1–18) and another lacking the AGE-binding cysteine-bound domain 62–78 (AdV-hLZ Δ ABCD), were constructed using the ExSite PCR-Based Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). The sequence of the constructs was confirmed by DNA sequencing. Constructs were cloned into the transfer vector pQBI-AdBM5-GFP (Adeno-Questkit, Qbiogene, Inc.). Recombinant adenoviruses were generated by *in vivo* homologous recombination, and the resultant titer was determined in transfected 293A cells according to the manufacturer's instructions. Recombinant adenoviruses were screened for LZ expression efficiency using the human lysozyme ELISA (Alpco).

Cell Culture and Cell Assays

The murine macrophage cell line TIB-186 (American Type Culture Collection, Rockville, MD) was maintained in Dulbecco's modified Eagle's medium, supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum. To examine the effect of ly-

sozyme overexpression on AGE turnover, confluent cells were transduced with the different adenoviral variants 24 hours before studies. For endocytosis and degradation, cell layers were incubated with ^{125}I -labeled AGE-BSA (B_{max} : 50 $\mu\text{g/ml}$, at 37°C for 2 hours), endocytosis was terminated with three washes of ice-cold phosphate-buffered saline (PBS), cell layers were dissolved in 0.1 mol/L NaOH, and radioactivity in the cellular fraction was determined. An aliquot from the media (50 μl) was trichloroacetic acid-precipitated (1:20 in PBS), and the radioactivity in the supernatant was counted to assess protein degradation.⁴⁰ All experiments were performed in triplicate.

Western Analysis

For Western blotting 10 μg of cellular protein or medium samples was heated at 95°C with Laemmli sample buffer containing 2% β -mercaptoethanol for 2 minutes and electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Separated proteins were then transferred onto nitrocellulose membranes. The membranes were blocked with 2% dry milk in PBS/Tween 20 for 1 hour) and then incubated with the primary antibody for 1 hour and with the appropriate peroxidase-conjugated secondary antibody (1:5000) for 1 hour. The bands were detected using the enhanced chemiluminescence method.

Cytokine mRNA and Protein Assessment

TIB-186 cells were grown in 100-mm Petri dishes. 24 hours after transduction with LZ constructs, cells were incubated with AGE-BSA, BSA (100 $\mu\text{g/ml}$), or LPS (50 ng/ml). Total RNA was extracted from cells after 24 hours of incubation for Northern blot analysis, and equal amounts of RNA (15 $\mu\text{g/lane}$) were run on a denaturing gel, transferred to nitrocellulose membranes, and probed with a ^{32}P -dCTP-labeled TNF- α -specific cDNA probe, as previously described.⁴³ RT-PCR was also conducted for TNF- α mRNA from TIB-186 cells using the primers as follows: forward, 5'-GTAGCCACGTCGTAGCAAACC-3' and reverse, 5'-CAGAGCAATGACTCCAAAGTAG-3' (430 bp). β -Actin was used as an RT-PCR control. TNF- α levels were determined by ELISA using a mouse TNF- α immunoassay kit (Biosource International).

TNF- α production was also determined by ELISA in freshly isolated peritoneal macrophages (5×10^5 cells/well) from LZ-Tg and WT (C57BL₆) mice stimulated with AGE-BSA (200 $\mu\text{g/ml}$) or LPS (1 $\mu\text{g/ml}$) for 24 hours ($n = 3/\text{group}$).⁴⁴ For assessment of the LZ dose response, macrophages from WT mice (5×10^5 cells/well) were pre-exposed to different concentrations of hen-egg lysozyme (100–300 $\mu\text{g/ml}$) for 2 hours before adding AGE-BSA (200 $\mu\text{g/ml}$), and TNF- α production in the media was assessed as above. Data from at least three experiments are expressed as mean $\pm$ SD $\mu\text{g/ml}$ TNF- α .

Generation of ApoE^(-/-) Mice with Hen-Egg Lysozyme (LZ) Transgene

Three-month-old C57BL/6J female mice, apolipoprotein E-deficient (ApoE^(-/-)) mice,^{23,26} and age-matched C57BL/6J mice transgenic for hen-egg lysozyme (LZ-Tg) were purchased from Jackson Laboratory (Bar Harbor, ME). ApoE^(-/-) mice were mated to LZ-Tg mice to create LZ/ApoE^(-/+) mice, which in turn were crossed to generate LZ/ApoE^(-/-) mice. Each subsequent generation was screened by PCR of genomic DNA using the following primers: for LZ-Tg, IMR0042 (5'-CTAGGCCACAGAATTGAAAGATCT-3'), IMR0043 (5'-GTAGGTGGAAATTCATGCATCATCC-3'), IMR0327 (5'-GAGCGTGAACTGCGCAAGA-3'), and IMR0328 (5'-TCGGTACCCTTGCGAGCGTT-3'), and for ApoE, IMR0180 (5'-GCCTAGCCGAGGGAGAGCCG-3'), IMR0181 (5'-TGTGACTTGGGAGCTCTGCAGC-3'), and IMR0182 (5'-GCCGCCCGACTGCATCT-3'). Serum hen-egg lysozyme and plasma cholesterol levels were determined in LZ/ApoE^(-/-) mice and compared with the levels in parental LZ-Tg and ApoE^(-/-) mice and in female C57BL/6 wild-type (WT) mice ($n = 6\text{--}8/\text{group}$). All groups were fed regular rodent chow (NIH-31) and had free access to water.

Femoral Artery Injury

Femoral artery injury was performed at 6 months as described.²⁶ In brief, mice were anesthetized and placed in the supine position with the lower extremities extended, a groin incision was made, and the segment of femoral artery between the epigastric and saphenous arteries was separated from the vein. An arteriotomy was made on the femoral artery, distal to the epigastric branch, a 0.25-mm-diameter angioplasty guide wire (Advanced Cardiovascular Systems, Temecula, CA) was introduced into the arterial lumen, the clamp removed, and the wire advanced and pulled back three times, each time reaching beyond the aortic bifurcation. The wire was then removed, and the arteriotomy site was ligated. The contralateral artery was 'sham-operated' and used as an uninjured control. Sham-operated arteries underwent dissection, temporary clamping, arteriotomy, and ligation, without passage of the wire. In this model, flow is maintained through the injured segment of femoral artery, because the epigastric artery and several muscular branches are preserved.

Evaluation of Aortic Atherosclerosis and Femoral Artery Injury Repair

Two months after arterial injury, mice ($n = 6/\text{group}$) were exsanguinated and perfusion-fixed in 4% paraformaldehyde (at 100 mmHg, 5 minutes).^{23,26} The heart and aorta were further fixed in 4% paraformaldehyde overnight, decalcified in 10% formic acid, and kept in 4% paraformaldehyde for 7 days. The heart was transected at the lower poles of the atria and processed for paraffin embedding, as described.²³ The aortic root was defined

anatomically as the portion of aorta extending cranially from the aortic sinuses to where the valve leaflets were no longer apparent in cross-section. Serial 5- μ m sections were taken from this area and stained with combined Masson elastic (CME) or with immunoreagents as indicated below. Thoracic aortas, between the subclavian branch and the renal arteries, were dissected, opened longitudinally, and examined at $\times 5$ magnification, and images were captured and evaluated using Image 1.6 software (National Institutes of Health).

For study of the femoral artery injury, the hind limbs and pelvis were excised en bloc, postfixed in 4% paraformaldehyde in PBS overnight, and decalcified in 10% formic acid for 12 hours. The thighs, containing the femoral vessels, were cut transversely, dividing the common femoral artery (~ 5 -mm length) in two segments. Specimens underwent standard dehydration, paraffin embedding, sequential sectioning, and mounting after CME and hematoxylin and eosin staining. Four sections (5 μ m each) from each artery were used for morphometry, and adjacent sections were used for immunohistochemistry.^{23,26}

Immunohistochemistry

Sections adjacent to those used for morphometry were stained for AGE, macrophages (anti-CD68), and α -smooth muscle actin, as described.^{23,26} Briefly, sections were boiled in 0.01 mol/L citric acid (pH 6.0) for 15 minutes for antigen retrieval and incubated with primary antibodies overnight at 4°C. After rinsing with PBS, biotinylated goat anti-rabbit IgG antibodies (1:500) were applied for 1 hour, followed by streptavidin peroxidase complexes (1:25) for 30 minutes. Diaminobenzidine was used as the final chromogen, and sections were counterstained for nuclei with hematoxylin. Negative controls were prepared by substituting the primary antibody with an irrelevant antibody.

Morphometry

Images of cross-sections of the femoral arteries and aortas from both groups of mice were captured using a video camera and digitized for analysis by Image 1.61 software (National Institutes of Health).^{23,26} Measurements of the luminal area, the area bound by the internal elastic lamina (corresponding to the luminal area in the absence of intimal lesions), and the area encircled by external elastic lamina (corresponding to overall vessel size) were obtained. The medial area (equal to the external elastic lamina area minus the internal elastic lamina area) and intimal area (equal to the internal elastic lamina area minus the luminal area) were then calculated.^{23,26} Two independent investigators, blinded to the study design, analyzed the sections by light microscopy.^{23,26}

Lysozyme, AGE, Isoprostane, Triglyceride, and Cholesterol Assays

LZ protein concentrations in transduced TIB-186 cells, extracts of aorta and heart tissues, or mouse serum were measured by ELISA, as described previously.⁴⁵ A standard curve was created using purified human or hen-egg LZ (0.07 to 40 ng/ml) as control substrates and anti-human (cross-reactive with mouse LZ) or anti-hen-egg LZ antibodies. Lysozyme mRNA was determined in multiple tissues by PCR, as described before.³⁷

AGE levels in serum were measured by competitive ELISA as described previously⁴⁰ and expressed as AGE units per milliliter.²⁵

Plasma 8-isoprostane levels were analyzed according to the manufacturer's protocol.⁴⁵ Briefly, fresh plasma (50 μ l), 8-isoprostane tracer, and STAT-8-isoprostane polyclonal antiserum (50 μ l) were added to 96-well plates and incubated at room temperature for 1 hour. The plates were then washed with AP buffer, and 200 μ l of *p*-nitrophenylphosphate in DEA buffer was added. The plates were allowed to develop in the dark for 60 to 90 minutes and were read at 415 nm with a microplate reader (Benchmark; Bio-Rad, Hercules, CA).

Plasma triglycerides were measured using the GPO-Trinder colorimetric assay kit (Sigma Diagnostics, St. Louis, MO) and expressed as milligrams/dl. Cholesterol levels were measured using an enzymatic immunoassay kit (Roche Applied Science, Indianapolis, IL) and expressed as milligrams/dl.

Statistics

All data are expressed as mean $\pm$ SD. Differences of means between groups were analyzed by the unpaired, two-tailed Student's *t*-test. Statistical significance was defined as a *P* value of <0.05 . All data analyses were performed using the GraphPad Prism statistical program (GraphPad Software, Inc., San Diego, CA).

Results

In Vitro Studies

The aims of the *in vitro* studies were twofold. The first was to determine whether overexpression of human lysozyme by macrophages led to enhanced uptake and degradation of AGE and increased AGE-induced cytokine induction. The second was to identify the domain(s) of the LZ polypeptide required for these effects on macrophages.

LZ AGE-Binding Domain

Enhanced LZ immunoreactivity was found by Western analysis of TIB-186 cells transduced with the full-length LZ (Adv-hLZ) or variants containing the AGE-binding domain 62–78 (ABCD) but lacking the secretory peptide 1–18 ((Adv-LZ)1–18), and those lacking the ABCD ((Adv-hLZ)-ABCD). In those transduced with GFP alone (Adv-GFP),

Figure 1. A: Expression of hLZ peptides following adenoviral vector (Adv) transduction of macrophage-like TIB-186 cells with hLZ human gene constructs. Adv-GFP, GFP alone; Adv-hLZ, full-length hLZ; Adv-hLZΔ1-18, variant lacking the signal peptide 1–18 of hLZ; Adv-hLZΔABCD, variant lacking the AGE-binding peptide 62–78 (ABCD) of hLZ; hrLZ, recombinant human LZ used as a positive control. Western blots of LZ peptides were performed with anti-hLZ antibody in cell extracts (1) or media (2), and GFP was assessed with anti-GFP antibody (3) in total cell extracts of Adv-transduced TIB-186 cells. Cellular uptake (B) and degradation (C) of ¹²⁵I-AGE-BSA and ¹²⁵I-BSA in TIB-186 cells transduced with adenoviral vector constructs, as indicated at the bottom of C. Data are shown as means ± SD of three experiments, each performed in triplicate. *P values between Adv-hLZ and Adv-hLZΔ1-18 versus control or Adv-GFP cells were <0.02 (B) and <0.005 (C), respectively.

only background LZ levels were detected (Figure 1A, row 1). LZ proteins were detected in media from cells transduced with constructs containing the LZ secretory peptide sequence but not from cells transduced with constructs lacking it (Figure 1A, row 2), while GFP vector was found in all Adv-transduced cell extracts (Figure 1A, row 3).

LZ Modifies AGE Uptake

When TIB-186 cells transduced with adenoviral-derived hLZ were incubated with ¹²⁵I-AGE-BSA, they incorporated ¹²⁵I-AGE-BSA. Cell-associated radioactivity was two- to threefold higher in cells transduced with the

Figure 2. Suppression of AGE-induced TNF-α in TIB-186 cells transfected with Adv-LZ. A: Northern blot analysis for TNF-α mRNA expression in control TIB-186 cells (first three lanes) or Adv-transduced with different LZ variants and incubated with AGE-BSA or BSA (100 μg/ml) or LPS (50 ng/ml) for 24 hours. **B:** RT-PCR analysis for TNF-α mRNA in control TIB-186 cells stimulated with AGE, BSA, PBS, or LPS, as indicated, or TIB-186 cells, transduced with LZ constructs and incubated with AGE-BSA. Data are represented as the mean ± SD, *P* < 0.02 (Adv-hLZ vs. Adv-hLZΔABCD). **C:** TNF-α production by control or LZ-construct transduced TIB-186 cells, stimulated with AGE (100 μg/ml) (black bars) or BSA (open bars). TNF-α production by control cells incubated with LPS alone (50 ng/ml) is shown as hatched bars. Data are shown as mean ± SD of three experiments, each performed in triplicate. *P < 0.02 versus control ± AGE.

Adv-LZ or Adv-hLZΔ1-18 variants compared to cells transduced with the vector lacking the ABCD motif (Adv-hLZΔABCD) or with the control variant Adv-GFP (*P* < 0.03, Figure 1B). In addition, degraded ¹²⁵I-AGE-BSA increased by twofold in the supernatant of TIB-186 cells transduced with the ABCD constructs (Adv-hLZ and Adv-hLZΔ1-18) but not in the supernatant of cells lacking ABCD (Adv-hLZΔABCD) or of cells with the vector control, Adv-GFP (*P* < 0.02) (Figure 1C).

Figure 3. A: LZ-Tg mouse peritoneal macrophages suppress AGE-induced TNF- α production. Non-elicited peritoneal macrophages (5×10^5 cells/well) from LZ-Tg and WT mice were stimulated with AGE-BSA (200 μ g/ml) or LPS (1 μ g/ml) for 24 hours, and TNF- α released into the medium was measured. Data are expressed as the amount of TNF- α produced by 5×10^5 macrophages. ** $P < 0.01$ vs. WT. **B:** Dose response of LZ inhibition: LZ-induced TNF- α inhibitory dose response in a dose-dependent manner in non-elicited peritoneal macrophages. Cells were preincubated with hen-egg lysozyme (100 to 300 μ g/ml, for 2 hours) before adding AGE-BSA (200 μ g/ml, for 24 hours). Data are expressed as a percentage, comparing TNF- α produced by AGE-stimulated macrophages not pre-exposed to LZ (black bars) to those exposed to increasing concentrations of LZ ($n = 3$ /group). Data from at least three experiments, each in triplicate, are shown as mean $\pm$ SD (picograms/ml). ** $P < 0.01$ vs. untreated; ## $P < 0.01$ vs. μ g/ml treated.

LZ Constructs Modulate AGE-Induced TNF- α Expression

The influence of AdV-LZ constructs on AGE-induced macrophage activation was assessed as TNF- α mRNA (Figure 2, A and B) and protein expression (Figure 2C). Based on Northern blot and RT-PCR analyses, AGE induction of TNF- α mRNA and protein was blocked in cells transduced with LZ variants containing the ABCD domain, ie, full-length LZ (AdV-LZ) and AdV-hLZ Δ 1–18 peptide (Figure 2, A and B). However, transduction with the variant lacking the ABCD motif (AdV-hLZ Δ ABCD) or with vector control, GFP (AdV-GFP), did not impair the synthesis of TNF- α mediated by AGE (Figure 2, A and B). The pattern of TNF- α protein expression followed that of mRNA (Figure 2C). Insulin-like growth factor-1 mRNA and protein expression in response to AGE were also studied and were found to mirror TNF- α responses (data not shown).

AGE-Induced TNF- α Expression Is Reduced in LZ-Tg Macrophages

To establish whether macrophages from LZ-Tg mice exhibit a similar response to AGE, TNF- α production was tested in peritoneal macrophages from LZ-Tg and WT

mice. AGE-stimulated LZ-Tg macrophages showed marked suppression of TNF- α relative to cells from WT mice ($P < 0.01$) (Figure 3A). Because LZ levels in LZ-Tg mice are fixed to a single LZ level, WT macrophages were cultured with increasing amounts of exogenous LZ before AGE stimulation (Figure 3B). LZ-treated macrophages demonstrated a dose-dependent TNF- α inhibitory response to AGE ($P < 0.01$). Thus, both exogenous and endogenous LZ suppressed AGE-mediated macrophage activation.

In Vivo Studies

Serum AGE and Peroxidation Products Are Reduced in LZ/ApoE^(-/-) Mice

At 3 months of age, serum hen-egg LZ was increased by three- to fivefold in the LZ/ApoE^(-/-) mice and LZ-Tg mice (Table 1). In age-matched ApoE^(-/-) mice expressing the LZ transgene (LZ/ApoE^(-/-)), serum AGE levels were reduced significantly (by 50%; $P < 0.02$, ApoE^(-/-) vs. LZ/ApoE^(-/-)) and were not different from those of WT mice (Table 1).

At 7.5 months of age, serum AGE levels were higher than at baseline in both the ApoE^(-/-) and the LZ/ApoE^(-/-) mice. However, in the LZ/ApoE^(-/-) mice

Table 1. Biochemical Characteristics

	ApoE ^(-/-)	LZ-Tg/ApoE ^(-/-)	LZ-Tg	C57BL6 (WT)
Cholesterol (mg/dl)	617 $\pm$ 144*	550 $\pm$ 131*	138 $\pm$ 6	140 $\pm$ 3
Triglycerides (mg/dl)	109.8 $\pm$ 6*	105.0 $\pm$ 8*	68 $\pm$ 3	72 $\pm$ 5
Serum AGE (U/ml) (at 3 months)	45 $\pm$ 8	18 $\pm$ 3†	22 $\pm$ 1.8†	30 $\pm$ 5
Serum AGE (U/ml) (at 7 months)	84 $\pm$ 9	38 $\pm$ 8.6†	30 $\pm$ 3†	44 $\pm$ 2.8
Serum lysozyme (ng/ml)	35 $\pm$ 2.0	125 $\pm$ 85‡	172 $\pm$ 80‡	42 $\pm$ 3
Plasma 8-isoprostane (pg/ml)	296 $\pm$ 85	168 $\pm$ 36‡	78 $\pm$ 28‡	110 $\pm$ 34

Data are means $\pm$ SD. Statistically significant values are shown as in the following footnotes.

*ApoE^(-/-) versus LZ-Tg and WT mice.

†LZ-Tg mice versus ApoE^(-/-) mice.

‡LZ-Tg mice versus ApoE^(-/-) and WT mice.

Figure 4. Tissue levels of hen-egg lysozyme (heLZ). **A:** Analysis of mRNA from tissues of heLZ-Tg mice by PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard. **B:** The amount of hen-egg lysozyme protein in extracts of heart and aorta was determined by ELISA (picograms/μg of protein). Note that the antibody does not cross-react with mouse LZ.

these remained significantly lower than in ApoE^(-/-) controls (Table 1). Importantly, AGE values in LZ/ApoE^(-/-) mice did not differ significantly from those in the WT group. Also, at 7.5 months of age, plasma levels of 8-isoprostane, a marker of oxidative stress, were reduced in both the LZ/ApoE^(-/-) mice and the LZ-Tg controls compared to the age-matched ApoE^(-/-) controls ($P < 0.05$) (Table 1). Consistent with previous studies, there were no significant differences in body weights, fasting blood glu-

cose levels, or renal function among the study groups (data not shown).

LZ mRNA and Protein Expression in Tissues of LZ-Tg Mice

LZ mRNA was expressed in multiple tissues (Figure 4A). Extracts of heart and aorta tissue, evaluated by ELISA, revealed that LZ protein was expressed in heart and aorta in LZ-Tg mice (Figure 4B). The amount in the aorta, on an LZ protein/mg of total protein basis, was more than threefold greater in aortic tissue compared to the heart.

Aortic Atherosclerosis Is Reduced in LZ/ApoE^(-/-) Mice

The severity of atherosclerosis of the aortic root at 7.5 months of age was significantly lower in LZ/ApoE^(-/-) mice (Figure 5A) compared to ApoE^(-/-) controls (Figure 5B) based on the size of the lesions. This was confirmed by determinations of the intimal to media ratio at the aortic root ($n = 6$, $P < 0.005$) (Figure 5C). In the LZ/ApoE^(-/-) mice the lesions were smaller, and the histological changes were less advanced, despite the fact that serum triglyceride and total cholesterol levels were not different between LZ/ApoE^(-/-) mice and ApoE^(-/-) controls (Table 1). Fibrous caps and superficial layering of foam cells were much less marked in the LZ/ApoE^(-/-) mice. The

Figure 5. Amelioration of aortic root atherosclerosis in LZ/ApoE^(-/-) mice. Aortic root sections (5-μm sections) from 7.5-month-old mice with (A) or without (B) the LZ-transgene were stained with CME (original magnification, ×100). LZ/ApoE^(-/-) mice have smaller lesions compared to the typical advanced atherosclerotic plaques of the ApoE^(-/-) group. **C:** Intimal to media ratio (I/M ratio) in aortic root segments obtained from both groups shown in A and B ($n = 6$ /group). Data are expressed as mean ± SD. * $P < 0.05$ versus ApoE^(-/-). **D** and **E:** Inflammatory cell infiltrate in aortic root sections, stained with anti-CD68 antibody, is reduced in LZ/ApoE^(-/-) mice. Representative sections stained with CME (original magnification, ×100).

Figure 6. Reduced thoracic aorta atherosclerosis in LZ/ApoE^{-/-} mice. **A:** Representative en-face images of atherosclerotic lesions in thoracic aorta from LZ/ApoE^{-/-} and ApoE^{-/-} mice. The panel shows images of aortas from both groups of mice (original magnification, $\times 5$). **B:** LZ overexpression reduces total area of thoracic aorta occupied by plaque. Calculated lesion percentage of the total aortic surface area ($n = 6/\text{group}$). Data are expressed as mean $\pm$ SD. * $P < 0.05$.

presence and abundance of inflammatory infiltrates was also significantly reduced in LZ/ApoE^{-/-} mice (Figure 5, D and E). In concert with the findings at the aortic root, the extent of atherosclerotic plaque formation along the thoracic aortas of LZ/ApoE^{-/-} mice was $\sim 80\%$ less than in ApoE^{-/-} controls ($P < 0.003$), based on both visual inspection (Figure 6A) and on measurement of the mean plaque surface area (Figure 6B).

Response to Femoral Artery Injury Was Reduced in the LZ/ApoE^{-/-} Mice

Five weeks after acute intraluminal injury, the femoral artery of the 7.5-month-old LZ/ApoE^{-/-} mice (Figure 7A) was widely patent, with a larger luminal area and smaller neointimal area than those of the ApoE^{-/-} mice (Figure 7B). The intimal to media ratio was significantly lower in the LZ/ApoE^{-/-}, compared with ApoE^{-/-} controls (1.15 ± 0.62 vs. 2.1 ± 0.72 , $P < 0.003$) (Figure 7C). Cross-sections of femoral arteries of ApoE^{-/-} controls 5 weeks after injury, as expected, showed significantly greater medial thickening than the contralateral uninjured arteries (data not shown).

Throughout the injured vessel wall of LZ/ApoE^{-/-} mice, there was reduced cellularity compared to ApoE^{-/-} controls (Figure 8a). AGE immunoreactivity was reduced and limited to the subintimal region in LZ/ApoE^{-/-} mice (Figure 8c), and there was no evidence of smooth muscle cell proliferation 5 weeks after endothelial denudation (Figure 8e). On the contrary, in the femoral arteries of ApoE^{-/-} controls, there was significant hypercellularity (Figure 8b) associated with the regions of AGE staining, which was distributed diffusely throughout the intima and media of the vessel wall (Figure 8d). In addition, there were large aggregates that stained positively for AGE in the media and adventitial layers, possibly reflecting insoluble AGE remnants. There was diffuse staining for α -smooth muscle actin in the subintima and media of ApoE^{-/-} controls (Figure 8f), coinciding with AGE staining, suggesting that the deposition of AGE was associated with proliferation of vascular smooth muscle cells. This finding was not present in the LZ/ApoE^{-/-} mice. The large, acellular aggregates of AGE staining may represent regions of dissolved lipid deposits (Figure 8f, arrow).

Discussion

This report demonstrates that hyperlipidemic, atherosclerosis-prone mice (ApoE^{-/-}) crossed with transgenic mice that overexpress LZ, an immune defense protein recently shown to reduce oxidant stress *in vitro* and *in vivo*, have reduced inflammatory and proliferative responses to acute arterial injury and decreased numbers of atherosclerotic plaques.^{23,26} The reduction in both acute and chronic vascular lesions, in the face of continued hyperlipidemia, was linked to an enhanced antioxidant balance manifest by a reduction in lipid peroxidation and proinflammatory molecules, such as AGE, and a decrease in inflammatory cell infiltrates in the vessel wall.

The means by which LZ modulates the antioxidant/oxidant balance may be related to its high affinity AGE binding (K_d 50 nmol/L), which recognizes at least two structurally distinct AGEs, N^ε-carboxymethyllysine and methylglyoxal derivatives, both of which have been identified in glycoxidized peptides and lipids.^{38,40} The AGE-binding site is a cysteine-rich domain at amino acids 62–78 (ABCD) consisting of a hydrophilic cysteine-bound domain.³⁸ LZ binding to AGE enhances AGE removal and turnover.^{37,39} In addition, the administration of LZ is associated with decreased AGE-elicited proinflammatory effects *in vitro* and *in vivo*,⁴⁰ by a mechanism linked to inhibition of oxidant stress.³⁷

Macrophages transfected with full-length lysozyme or with LZ constructs containing ABCD showed enhanced uptake and degradation of AGE proteins and blunting of AGE-induced intracellular TNF- α synthesis, whereas those with LZ constructs lacking the ABCD motif did not exhibit these properties. A similar decrease in TNF- α secretion was found in macrophages from mice transgenic for LZ or when LZ was added to normal macrophages. Thus, the anti-inflammatory and antiproliferative properties of LZ in macrophages appeared to depend on the presence of the ABCD motif.

Crosses between hyperlipidemic mice (ApoE^{-/-})^{23,26} and LZ transgenic mice⁴¹ (LZ/ApoE^{-/-} mice) were developed to study the effect of endogenous source of LZ on vascular disease. The choice of this model was based on our previous study showing that mice transgenic for LZ (LZ-Tg) are resistant to the induction of both acute and chronic oxidant stress induced by environmental oxi-

Figure 7. Arterial wall response to acute injury and restenosis is suppressed in LZ/ApoE^{-/-} mice. Light photomicrographs of femoral arteries cross-sections, 5 weeks after endothelial denudation. **A:** LZ/ApoE^{-/-}. **B:** ApoE^{-/-}, CME-stained injured arteries. **C:** Calculated value of the intimal to media ratio (I/M ratio) in sections from ApoE^{-/-} with and without LZ transgene, respectively (*n* = 6/group). Data are expressed as mean ± SD. *P < 0.05. Original magnification, ×200.

dants.³⁷ Interestingly, these mice also exhibited elevated baseline antioxidant balance in blood and liver tissue, based on increased reduced glutathione, and lowered serum AGE levels.

Therefore, we reasoned that the increased LZ levels in the LZ/ApoE^{-/-} mice would lead to decreased levels of AGE and oxidant stress, which would suppress the femoral artery vascular responses to injury and the number of atherosclerotic lesions in the aorta. LZ/ApoE^{-/-} mice had increased circulating LZ levels, decreased serum

Figure 8. Cellularity, AGE deposits, and SMC proliferation are reduced in acutely injured femoral arteries of LZ/ApoE^{-/-} mice. **a** and **b:** Hematoxylin and eosin staining. **c** and **d:** AGE immunostaining. **e** and **f:** α-SMC actin immunostaining. **Arrow** in **e** points to the area of subendothelial SMCs corresponding to intracellular AGE deposits (**arrow** in **c**). **Arrow** in **f** points to area free of SMC corresponding to extracellular AGE aggregates (**arrow** in **d**). Original magnification, ×200.

AGE levels, and a significant reduction in endogenous lipid peroxide generation, consistent with suppression of systemic oxidant stress. These observations were independent of continued hyperlipidemia. Furthermore, the LZ/ApoE^{-/-} mice showed a significant reduction in the degree of atherosclerotic lesions at the aortic root and a greater than 80% decrease in the total surface area of the thoracic aorta covered by plaques. Fibrous caps and inflammatory infiltrates were also significantly less prominent. Thus, high LZ levels contributed to suppression of circulating oxidants and attenuation of the inflammatory lesions. The atheromas present in the LZ/ApoE^{-/-} mice might be attributed to oversaturation of LZ by oxidants. Namely, LZ levels remained constant over time in the LZ/ApoE^{-/-} mice, whereas the levels of oxidants, such as AGEs/ALEs, continued to increase. The inflammatory response to acute vascular injury was also markedly suppressed in the LZ/ApoE^{-/-} mice, and there was a marked decrease in the number of proliferating smooth muscle cells (SMCs) and in overall cellularity of the arterial wall. These data emphasize the fact that the sustained reduction in oxidant stress, due to lower AGE and 8-isoprostane levels, was associated with high levels of LZ and that these factors contributed to a reduction in

atherosclerosis and in the severity of the femoral artery response to acute injury, regardless of sustained hyperlipidemia.

It is not known whether LZ interacts with cholesterol, triglycerides, or other metabolites. However, LZ binds AGEs/ALEs,^{38,40} molecules that induce chronic oxidative stress and favor pathological vascular responses to mechanical injury.^{24,28} AGEs were shown to be involved in vascular injury in high fat-fed C57B6 and ApoE^(-/-) mice.⁴⁷⁻⁴⁹ Increased AGE deposits have also been described in the arterial wall of diabetic subjects.⁴⁹ These aortic deposits have been thought to be enhanced by both hyperglycemia and hyperlipidemia.^{24,28} In the current study, the extent of the aortic surface covered by plaques and the severity of aortic root atherosclerosis paralleled the levels of both circulating and tissue-deposited AGE derivatives as well as with circulating lipid peroxidation products, in the absence of other metabolic differences between the groups, eg, in glucose or lipid levels. Thus, the suppression of chronic lesions and the diminution of the inflammatory and proliferative response to acute arterial injury were both related to the reduction of systemic oxidant stress burden, which accompanied the high levels of lysozyme production in the LZ/ApoE^(-/-) mice.

The pathway by which LZ blocks AGE-induced oxidant stress and downstream inflammatory cytokine production, ie, TNF- α , in macrophages is unknown. However, our data are consistent with previous evidence showing that AGE-mediated NF- κ B responses are inhibited in different cell types by the addition of LZ.³⁹ More recently, we found that the addition of LZ blocks AGE-mediated responses *in vivo* and *in vitro*, as well as non-AGE ROS-mediated activation of genes related to stress response, ie, p66 and c-Jun.^{37,50,51}

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