Amelioration of oxidant stress by the defensin lysozyme

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Liu, Huixian, Feng Zheng, Qi Cao, Bin Ren, Li Zhu, Gary Striker, and Helen Vlassara. Amelioration of oxidant stress by the defensin lysozyme. Am J Physiol Endocrinol Metab 290: E824–E832, 2006. First published November 29, 2005; doi:10.1152/ajpendo.00349.2005.—Reactive oxidant species (ROS), products of normal metabolism, cause oxidant injury if they accumulate in pathological amounts. Lysozyme (LZ) contains an 18-amino acid domain that binds agents such as advanced glycation end products (AGE) that generate ROS. We examined whether endogenous LZ affected physiological, or baseline, antioxidant balance and provided protection against both acute and chronic oxidant injury, using paraquat and H$_2$O$_2$ as agents of acute injury and AGE for chronic injury. Hen egg LZ-Tg mice had threefold higher serum LZ levels and decreased baseline AGE levels in serum and liver. These findings were linked to an enhanced baseline systemic GSH-to-GSSG ratio. Baseline levels of stress response genes p66$^{Shc}$ and c-Jun were also lower in liver tissue of LZ-Tg mice. Survival from severe oxidant injury induced by paraquat was twofold greater in LZ-Tg mice. In addition, LZ-Tg mice were resistant to chronic exogenous oxidant stress (OS) induced by AGE administration. Preincubation of hepatocytes (Hep G2) with LZ suppressed redox balance at baseline, as well as OS after added paraquat, AGE, or H$_2$O$_2$. LZ also ameliorated paraquat-enhanced cell apoptosis in a dose-dependent manner and suppressed AGE-induced p66$^{Shc}$ expression and c-Jun phosphorylation in Hep G2 cells. Thus LZ provides protection against acute and chronic oxidant injury by mechanisms involving suppression of OS generation and of OS response genes.

reactive oxygen species; paraquat; glycoxidation; lipoxidation; redox balance; oxidant injury

UNDER NORMAL CONDITIONS, where the levels of reactive oxygen species (ROS) are tightly regulated and play a role in normal metabolism, the body’s antioxidant defenses are able to successfully manage nonpathological amounts of exogenous or endogenous oxidants from reactive oxygen/nitrogen species (43). The environment can be a source of excess oxidants, which can cause both acute and chronic cellular injury (31, 43, 53). Exposure to a number of environmental agents, i.e., herbicides, radiation, air pollution, or glycation and oxidation products contained in foods, can reduce antioxidant reserves and lead to acute or chronic injury characterized by elevated oxidative stress (OS) (11, 21, 31, 43, 49). An example of a source of acute OS is the widely used herbicide, 1,1’dimethyl-4,4’-bipyrindimine [paraquat (PQ)], which causes severe, acute, and/or chronic neurotoxicity due to ROS production, c-Jun phosphorylation, and activation of caspase-3 (33, 44). Peptides or lipids contained in foods are another common source of oxidants causing chronic OS injury, since they contain free NH$_2$ groups that can be spontaneously modified by reducing sugars or fats, to form bioactive and prooxidant derivatives called advanced glycation end products (AGE) or advanced lipoxidation end products (ALE) (5, 18). These end products are ubiquitously present in food, as part of the normal dietary environment, and are important contributors to ROS production (5) and to chronic OS in aging humans and animals (31, 51, 52). The usual diet of animals and humans contains excess AGE and ALE, and these oxidants contribute to the total body oxidant load (11, 21, 59). Constant exposure to dietary oxidants results in a loss of normal antioxidant reserves, rendering animals more susceptible to chronic inflammatory states (5).

Interestingly, restriction of dietary oxidants restored normal antioxidant homeostasis and interfered with the development of some diseases, which are common in aging animals, such as diabetes, chronic cardiovascular disease (CVD), and chronic kidney disease (CKD) (16, 26, 59). These data suggest that antioxidant homeostasis may be compromised in animals and humans under conditions that have previously been considered to be normal or “baseline”. This may require a reevaluation of the levels of OS at baseline. Antioxidant homeostasis is also regulated by the cellular uptake and detoxification of oxidants, such as AGE, by the various types of cellular AGE receptors. Some AGE receptors, such as RAGE, promote AGE-induced OS (19, 39, 55, 56), whereas others, such as AGE-R1, counteract these responses (14, 16, 27).

AGE are progressively elevated with aging and are also high in a number of common diseases, including diabetes mellitus, CVD, and CKD (3, 4, 34, 35, 38, 48, 51, 57, 58). These conditions are currently thought of as subacute inflammatory states due, in part, to elevated OS. Some aspects of AGE-induced OS have been attributed to an imbalance between proinflammatory AGE actions and impaired antioxidant defenses, a postulate supported by experimental interventions that reduce AGE-induced OS (2, 9, 41). Thus inhibition of AGE-related OS may reduce one source of oxidant injury, an effect that may slow or arrest the decline of end-organ function associated with high levels of OS (2, 9, 13, 25, 42).

Lysozyme (LZ) is among the lesser known soluble molecules that participate in AGE “detoxification” (24, 29, 58). LZ is a member of a well-characterized family of native, highly conserved host-defense proteins called defensins, which exist in several isoforms and are distributed widely in biological fluids and tissues and have antibacterial activity (8). LZ has recently been found to exhibit high AGE-binding affinity (K$_d$ 50 nM), recognizing at least two structurally distinct peptide-linked AGE, A$^N$-carboxymethyllysine and methylglyoxal derivatives (29, 58). The LZ AGE-binding site is localized to the NH$_2$-terminal cysteine loop, consisting of a hydrophilic cysteine-bounded domain, which overlaps with the domain of the...
bactericidal activity of this family of proteins (24). LZ binding to AGE was initially found to enhance AGE removal and turnover. In addition, LZ was shown to decrease AGE or endotoxin-elicited proinflammatory effects in vitro and in vivo, via a mechanism that was not further defined (45–47, 58).

Since changes in physiological antioxidant balance or oxidant stress are manifest in both acute and chronic injury (43), we explored the impact of endogenous LZ on agents that produce either severe acute (PQ and H2O2) or chronic, low-grade OS (AGE). We studied these responses in vivo, using transgenic mice expressing hen egg LZ (LZ-Tg mice) and in vitro using hepatoma cells HepG2. The results show that LZ raises the levels of normal antioxidant reserves and confers resistance to molecules that cause either acute or chronic OS and that this may occur via negative regulation of stress response genes.

MATERIALS AND METHODS

Animals. LZ-Tg male mice, C57BL6/12TgN (Mlg5HEL) 5Ccg (cat no. 002598), which express hen egg (he)LZ gene under the control of a metallothionein-1 promoter, and age-and sex-matched wild-type (WT) C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) (1). Mice were maintained in the animal facility of Mount Sinai School of Medicine under specific pathogen-free conditions. After death, blood was obtained by cardiac puncture, the aorta was cannulated, the inferior vena cava was opened, and physiological saline was perfused at normal arterial pressure until the liver blanched.

The presence of the transgene was identified by polymerase chain reaction of genomic DNA as previously described (Ref. 1; http://jaxmice.jax.org). All experiments were performed in 5-mo-old LZ-Tg mice and WT mice. Experiments were conducted in accordance with the guidelines for the care and use of animals approved by the Institution Animal Care and Use Committee, Mount Sinai School of Medicine.

LZ and AGE assays. Serum and urine concentrations of heLZ were measured by enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-heLZ (Accurate Chemical, Whitewater, WI) or a sheep anti-human LZ polyclonal antibody (cross-reactive with mouse LZ: Chemicon International, Temecula, CA). The reaction was revealed with a biotin-conjugated secondary antibody and alkaline phosphatase substrate. A standard curve was created using purified human or heLZ (0.07–40 ng/ml).

Serum, tissue AGE levels, and urinary low-molecular-weight AGE peptides were measured by a competitive ELISA, based on an anti-Nε-carboxymethyllysine monoclonal antibody (4G9), as previously described (30). Urinary AGE peptide excretion was corrected against urinary creatinine levels and expressed as AGE-to-creatinine ratio (58). AGE-modified [AGE-bovine serum albumin (BSA)] and native BSA were prepared from endotoxin-free BSA (fraction V; Sigma Aldrich, St. Louis, MO), as described, and used only if it contained <0.012 ng/ml endotoxin (based on Limulus Amebocyte Lysate Pyrogent assay) (58).

Glutathione and oxidized glutathione assay. Serum and tissue GSH and GSSG levels were measured using a kit from OXIS International (Biotestech GSH/GSSG-412, Portland, OR). For GSH assay, 50 μl of the sample were mixed with 350 μl of 5% metaphosphoric acid. After centrifugation, supernatants were diluted 1:61 with the assay buffer and reacted with equal volume of chromogen, glutathione reductase, and nicotinamide adenine dinucleotide phosphate. Changes in absorbance were recorded at 412 nm with a spectrophotometer. For GSSG assay, 100-μl samples were reacted with 290 μl of 5% metaphosphoric acid. After centrifugation, supernatants were diluted 1:15 with GSSG buffer. Diluted samples were incubated with an equal volume of chromogen, glutathione reductase, and nicotinamide adenine dinucleotide phosphate. Changes in absorbance at 412 nm were monitored for 3 min, and the results were calculated based on standard curves, created with known amounts of GSSG, as described (5).

In vivo induction of OS by PQ and AGE. LZ-Tg mice or WT male mice (5 mo old, n = 18/group) were injected with 1,1′-dimethyl-4,4′-bipyridinium (PQ, 70 mg/kg ip) (Sigma). Also, AGE-BSA (40 μg/g; 1 day−1 ip for 10 days), a ROS-generating agent, was administered to LZ-Tg mice or WT mice (n = 6/group). Blood, urine, and liver tissue were collected at death for determination of AGE and GSH-to-GSSG ratio (GSH/GSSG) levels. Survival was monitored for up to 80 h after PQ injection.

Induction of OS by PQ, H2O2, or AGE in Hep G2 cells. Cells from the liver hepatoma cell line, Hep G2, were cultured with RPMI 1640 medium containing 10% fetal bovine serum. To test the effect of LZ on different OS-promoting agents, e.g., PQ, H2O2, or AGE, Hep G2 cells were seeded in 24-well plates (5 × 104/well). In some wells, cells were preincubated with 200 μg/ml (or as indicated) of HEL (hen egg lysozyme) or BSA 1 h before exposure to either AGE-BSA, BSA, H2O2 (50 μM), or PQ (0.2–1 mM). Cells were collected 20 h (or as indicated in Figs. 1–8) after exposure. GSH and GSSG levels were measured in cell lysates, as described above. ROS was measured as described (6). Briefly, superoxide generation was determined by the addition of hydroethidine (at a final concentration of 10 μM) to cell layers that had been exposed to H2O2 for 45 min. Since hydroethidine is oxidized to O2, the loss of fluorescence is directly proportional to the amount of superoxide generated. Hydroethidine fluorescence was measured at 352 nm for excitation and 434 nm for emission (6). The controls included cells pretreated with catalase (1,000 U/ml) before the addition of H2O2.

Cell apoptosis was assessed using a Cell Death ELISA kit (Roche Diagnostics). Briefly, microplate wells were first coated with anti-histone antibody at room temperature for 1 h. After blocking, sample was added to the well. DNA fragments in the lysate were detected by peroxidase-conjugated anti-DNA antibody and peroxidase substrate.

Ligand blot. Serum samples (20 μl) collected from the LZ-Tg mice and WT controls (n = 5/group) were mixed with an equal volume of sample buffer containing 2% β-mercaptoethanol and heated at 95°C for 5 min before loading on 12% PAGE. After electrophoresis, the gels were transferred to nitrocellulose membranes and blocked with 2% BSA in phosphate buffer solution (PBS) for 1 h. After they were washed twice with PBS containing 0.05% Tween 20, the membranes were probed with 125I-labeled AGE-BSA (4 × 106 cpm) for 1 h in the presence or absence of unlabeled AGE-BSA (100-fold excess). The blots were washed extensively with PBS-Tween 20, air dried, and exposed to XAR film (Kodak, Rochester, NY) at −80°C. The intensity of specific bands was quantitated by computer-assisted densitometric analysis (Bio-Rad Laboratories, Hercules, CA). Ligand blots were performed at least three times for each mouse group.

Western blot analysis. For Western blotting, proteins were extracted from intact mouse liver or cultured hepatocytes (Hep G2). Equal amounts of protein were loaded onto the gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in PBS-Tween 20 solution for 1 h, washed twice, and incubated with rabbit anti-mouse c-Jun (L0203, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-c-Jun (Cell Signaling Technology, Beverly, MA), or rabbit anti-Shp6 (anti-p66Shc) (BD Biosciences Pharmingen, San Diego, CA). Membranes were rehybridized with rabbit anti-mouse tubulin antibody (Sigma) to ensure equal protein loading. The reactions were revealed with a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). The intensity of specific hybridized bands was quantitated by densitometry.

Statistical analysis. Values shown represent the means and SD of at least three independent measurements. Two-tailed unpaired t-test was used to evaluate differences between LZ-Tg mice and WT mice. Significance was chosen at P < 0.05. Survival data of animals injected with PQ were analyzed by the Kaplan-Meier method.
RESULTS

Overexpression of LZ is associated with decreased serum and tissue AGE levels and enhanced renal AGE excretion in LZ-Tg mice. As expected, serum LZ levels were 3.2-fold higher in LZ-Tg mice than in WT mice [LZ-Tg mice, n = 8, 169.4 ng/ml (SD 47.4) vs. WT, n = 8, 52.8 ng/ml (SD 6.2), P < 0.05]. Of interest, this was associated with significantly lower baseline AGE levels in serum (by 56%, P < 0.01) and in liver (by 30%, P < 0.01) (Fig. 1A), while the baseline of urinary AGE-to-creatinine ratio in LZ-Tg mice was approximately threefold greater than in WT mice (P < 0.05; Fig. 1A). Urinary albumin and creatinine excretion were normal in both groups (data not shown). Consistent with previous data (26), LZ-enriched sera from different LZ-Tg mice exhibited a two- to fourfold greater binding capacity for 125I-AGE-BSA, compared with sera obtained from WT mice (Fig. 1B, lanes 1–6, and C). The specificity of AGE binding was demonstrated by competitive inhibition in the presence of excess unlabeled AGE-BSA (Fig. 1B, lane 7).

Redox status is elevated in LZ-Tg mice. The association between elevated LZ levels and the reduced serum AGE levels with the redox status in LZ-Tg mice, as reflected by the total glutathione levels (reduced GSH and oxidized GSSG), was assessed in both transgenic and WT mice under physiological (or baseline) conditions. There was a significantly higher GSH/GSSG in whole blood (55%, P < 0.01) and liver tissue (116%, P < 0.01) in LZ transgenic than in WT mice (Fig. 2).

Exogenous AGE induces systemic oxidant stress, which is ameliorated in LZ-Tg mice. LZ-Tg mice and WT mice were infused with AGE-BSA (40 μg·g⁻¹·day⁻¹·ip) for 10 days, to examine the extent to which a sustained systemic increase in AGE alters the redox status. Following AGE infusion, a three- to fourfold elevation of AGE levels from baseline was noted in all groups; however, the maximal rise was significantly smaller in serum of LZ-Tg [60 U/ml (SD 3.7)] compared with serum from WT [118 U/ml (SD 28)] (P < 0.01; Fig. 3A). AGE levels in liver tissue of LZ-Tg mice also remained lower [28 U/mg (SD 3)] than that in liver tissue from WT mice [43 U/mg (SD 5), P < 0.01]. As noted previously (58), there was a fourfold increase in urinary AGE-peptide excretion, observed in LZ-Tg (P < 0.05; Fig. 3B). The increased systemic AGE levels after AGE treatment were associated with a reduction in blood and liver GSH/GSSG in both WT and LZ-Tg mice (Fig. 3C). However, in the LZ-Tg mice, these ratios remained significantly higher than in the AGE-challenged WT mice (P < 0.05; Fig. 3D).

LZ-Tg mice have increased resistance to PQ-induced OS and death. Whether the resistance to OS conferred by LZ extended to stimuli other than AGE was examined in LZ-Tg mice.
mice that were given a single dose of PQ, a neurotoxic herbicide, which causes death via severe acute OS when given at a high dose (44). LZ-Tg mice lived significantly longer than WT mice after PQ injection (Fig. 4). At the 50% lethal dose for WT mice (median lifespan: 30 h) >85% of LZ-Tg mice were alive (median lifespan: 60 h; \( P < 0.01 \)). In addition, 22.2% of LZ-Tg mice survived up to 2 mo, whereas none of the WT mice survived more than 60 h.

**LZ suppresses OS induced by \( \text{H}_2\text{O}_2 \), AGE, or PQ in Hep G2 cells.** The effect of exogenous LZ on oxidant balance in cells challenged by AGE, PQ, or \( \text{H}_2\text{O}_2 \) was directly assessed by pretreating Hep G2 cells with LZ (200 \( \mu \text{g/ml} \) for 1 h) and then exposing them to increasing doses of AGE-BSA or BSA (50–300 \( \mu \text{g/ml} \)), PQ (0.2–1 mM), or \( \text{H}_2\text{O}_2 \) for 20 h. The addition of AGE or PQ reduced GSH/GSSG levels below baseline levels in Hep G2 cells not treated by LZ in a dose-dependent manner; however, this effect was largely blocked by the addition of exogenous LZ (Fig. 5A). Similar results were noted with \( \text{H}_2\text{O}_2 \). Pretreatment with LZ or catalase largely prevented the reduction of GSH/GSSG by \( \text{H}_2\text{O}_2 \) (data not shown). As expected, the addition of \( \text{H}_2\text{O}_2 \) increased intracellular superoxide generation. Pretreating cells with LZ reduced \( \text{H}_2\text{O}_2 \)-induced superoxide production (Fig. 5B). LZ also blocked PQ-stimulated apoptotic cell death in a dose-dependent manner (\( P < 0.02 \)) (Fig. 5C). Interestingly, the addition of LZ maintained or raised the constitutive GSH/GSSG in Hep G2 cells unchallenged by oxidants (\( P < 0.05 \)) (Fig. 5A).

**LZ decreases p66Shc and c-Jun expression and activity levels in LZ-Tg mouse liver and Hep G2 cells.** Increased cellular OS response has been linked to numerous genes, including p66Shc and c-Jun (28, 32, 33). We examined the levels of both p66Shc and c-Jun.

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**Fig. 2.** LZ-Tg mice exhibit elevated baseline systemic antioxidant balance in the blood and liver tissue. Blood and liver GSH and GSSG levels were measured in resting LZ-Tg and WT mice (\( n = 8 \)/group). Data are expressed as means and SD of the ratio of GSH to GSSG levels, performed in triplicate. \( P \) values are shown.

**Fig. 3.** LZ-Tg mice maintain lower systemic AGE levels and higher antioxidative balance than WT mice, after receiving exogenous AGE for 10 days. LZ-Tg and WT mice (\( n = 8 \)/group) were infused with AGE-BSA (40 \( \mu \text{g} \cdot \text{g}^{-1} \cdot \text{day}^{-1} \) ip) for 10 days. Serum and liver tissue levels (A), urinary AGE-peptide levels (B), and GSH-to-GSSG ratios (C) were measured, as above. Data are shown as means and SD of 3 independent tests/group, each in triplicate. \( P \) values are shown.

**Fig. 4.** Twofold greater survival of LZ-Tg mice after severe acute oxidant injury induced by paraquat (PQ). Kaplan-Meier analysis is shown of cumulative survival data from LZ-Tg and WT mice (\( n = 18 \)/group) injected with a single lethal dose of PQ (70 mg/kg ip). *\( P < 0.05 \), LZ-Tg vs. WT.
and c-Jun protein under baseline conditions in liver tissue and found them significantly lower in LZ-Tg mice than in WT mice (P < 0.025 and <0.05) (Fig. 6).

The in vitro response of liver (Hep G2) cells paralleled the in vivo findings in liver tissue in that cells pretreated with LZ (LZ+, P < 0.05; Fig. 7). Importantly, LZ pretreatment prevented the increase in p66Shc levels in Hep G2 cells stimulated with AGE (P < 0.01; Fig. 7). Furthermore, preincubation of cells with LZ blocked the AGE-induced Tyr phosphorylation of c-Jun (Fig. 8, A and B) (23). The phosphorylation of c-Jun peaked rapidly after AGE stimulation (by 5–10 min) in Hep G2 cells, but this effect was completely blocked by LZ pretreatment. (Fig. 8C).

DISCUSSION

Although normal levels of ROS are a part of normal metabolism, when present in excess or if antioxidant defenses are compromised, they can contribute to OS. There is considerable evidence that a sustained oxidant imbalance is a determinant of mammalian cellular injury and organ damage, leading to several disease states and to the acceleration of aging (22, 37, 40, 50).

The main findings presented in this report are fourfold. First, LZ raises baseline intracellular and systemic antioxidant reserves. This effect may be partly mediated by a reduction in the amount of oxidants present at baseline, or by a direct effect of LZ on ROS metabolism, as suggested by our finding that LZ blocked OS induced by H2O2. Second, LZ confers resistance to PQ, an agent that induces acute OS at high doses. Namely, it blocks cellular apoptosis in vitro and reduces mortality in vivo. Third, LZ confers protection against chronic OS induced by AGE derived from endogenous or exogenous sources. Finally, the antioxidant properties of LZ are partly mediated by a reduction of ROS levels and of stress response genes, including p66 and c-Jun (28, 32, 33). These data reveal, for the first time, an inverse relationship between the state of innate redox balance and systemic levels of exogenous oxidants, of which AGE represent only one example. As with many environmental oxidants, AGE are an important source of intracellular as well as of systemic ROS (36, 41).

Our laboratory previously found that the administration of LZ enhanced AGE removal and suppressed AGE-induced OS and the associated inflammatory responses in vivo and in vitro (58). We used mice that overexpressed the avian LZ transgene (LZ-Tg mice) to address the question of whether endogenously
produced LZ also suppresses OS and of its mechanism of action (1). An unexpected finding in the LZ-Tg mice was that the levels of molecules linked to chronic OS and the levels of AGE were reduced at baseline. Namely, the baseline serum and liver AGE levels were significantly lower in LZ-Tg mice than in nontransgenic controls, while there was a marked enhancement of the resting redox state (approximately twofold higher GSH/GSSG), compared with that in nontransgenic controls. Coupled with the observations that LZ pretreatment resulted in resistance to H2O2-induced changes in intracellular superoxide levels in Hep G2 cells, these data suggest that LZ has a direct effect on ROS levels in vivo and in vitro. Taken together, these data suggest that, while it has been assumed that unchallenged mice and cells in vitro are not in a state of OS under baseline conditions, there may exist unrecognized exogenously derived compounds, which raise the steady-state levels of ROS. Thus partial compromise of antioxidant balance by transient or chronic elevations in OS may be the rule in mice and cells in culture, which had heretofore been considered to be in an unchallenged or resting state. While the reasons for this finding may be complex, the environment is an important source of

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**Fig. 6.** Baseline expression levels of p66 and c-Jun are reduced in LZ-Tg mice. 
*Panel A:* representative Western blots of 2 LZ-Tg and 2 WT mouse liver tissue extracts. After blotting with anti-p66 or anti-c-Jun antibody, membranes were probed with anti-tubulin antibody to ensure equal protein loading. *Panel B:* densitometric analysis of Western blots (n = 6 mice/group tested). Data are from 3 independent experiments, each of 2 mice, expressed as test protein-to-tubulin ratio. P values are as indicated.

**Fig. 7.** Exogenous LZ suppresses p66 levels in Hep G2 cells stimulated by AGE in vitro. 
*Panel A:* Western blot of cellular extracts with anti-p66. *Panel B:* densitometric analysis of Western blots. *Panel C:* time course of c-Jun Tyr phosphorylation, after stimulation of LZ-pretreated (200 μg/ml × 1 h) or untreated Hep G2 cells with AGE-BSA (100 μg/ml) for the indicated time intervals and assessed by Western analysis.
oxidants (31, 43, 53), including those from the diet or the serum used in tissue culture (11, 31, 43). This concept must be evaluated by other methodologies and should be the subject of further investigation.

The current data also showed that exogenous and endogenous LZ conferred significant resistance to elevated OS. This was examined using high-dose PQ, a potent inducer of acute OS (33, 44). Exogenous LZ blocked PQ-induced apoptosis and cell death in cultured Hep G2 cells. The LZ protection against acute OS by PQ in vitro was mirrored by the high levels of endogenously produced LZ in vivo, i.e., mortality was reduced by >50% in LZ-Tg mice injected with a lethal dose of PQ (200% greater than in controls). Of note is the fact that the rate of survival of LZ-Tg mice exceeded that reported in other mouse models that demonstrate PQ resistance, e.g., mice deficient in insulin-like growth factor I receptor (IGF-IR−/−, ~20% greater than in controls) (10, 17). In addition, other studies show that there is an amelioration of the response to the administration PQ at high levels, both in vivo and in vitro, in glutathione peroxidase knockout mice, showing that the generation of ROS underlies the OS (7). These data and those in the current experiments provide evidence that LZ reduces cellular ROS levels. The PQ model is pertinent not only because it is present in the environment, but also because the pathways activated by the IGF-IR include the MAPK/ERK1/2 and Akt kinase signaling cascades, pathways that are also implicated in cellular AGE signaling, via receptors such as RAGE, and NADPH oxidase (55, 56). Regulatory proteins acting downstream from IGF-IR, such as the Shc isoform p66Shc or the activator protein-1 c-Jun, are also redox-responsive downstream from IGF-IR, such as the Shc isoform p66Shc or the activator protein-1 c-Jun, are also redox-responsive factors that can be induced by agents such as PQ (33). Oxidants induce phosphorylation of p66Shc at Ser36, which, in turn, suppresses forkhead transcription factor, an important regulator of key antioxidant systems such as superoxide dismutase and catalase (28, 32). Both overexpression of catalase and mutations in p66Shc have been shown to extend lifespan in mice (28, 40). Interestingly, baseline levels of p66Shc and c-Jun were higher in liver tissue of controls, compared with resting LZ-Tg mice in our studies, suggesting that there was an elevated OS state under “normal or baseline conditions” in control mice.

Endogenous LZ also provided a significant degree of protection against long-term exposure to an exogenous source of AGE that are a known cause of moderate OS. LZ-Tg mice that had received AGE for 10 days had only a minimal rise in OS and small changes in stress response genes such as p66Shc and c-Jun, whereas there were marked increases in WT controls. These in vivo data were extended by in vitro studies, in which the addition of exogenous LZ to AGE-stimulated Hep G2 cells blocked the expected rise in cellular OS, apparently via pathways that involve reduced activation of p66Shc and c-Jun (54).

One of the mechanisms by which LZ protects against AGE-induced OS in vivo might be by blocking the activation of receptors or signal molecules proximal to the cell surface, such as the p66Shc adaptor protein. Unlike receptors that involve stress response genes, such as IGF-IR (17), LZ is not known to bind to a specific cell-surface receptor. In the case of AGE, LZ is thought to form LZ-AGE complexes that are taken up by endocytosis, possibly via AGE or scavenger receptors (30, 52, 58). It is unlikely that the LZ effects involve RAGE, a prooxidant and nonendocytic AGE receptor (4, 19, 56). However, the involvement of AGE-R1, a receptor shown to enhance AGE endocytosis and to suppress the prooxidant effects of AGE, cannot be ruled out (27). Moreover, receptor- and nonreceptor-dependent mechanisms of LZ action against oxidants such as H2O2 or PQ may be involved and warrant further investigation.

Based on the current and previous studies, LZ appears to lower OS associated with an increased exogenous AGE burden by two means. First, LZ overexpression increases cellular endocytosis and degradation of AGE-BSA (58). Second, endogenous LZ enhances the renal clearance of AGE peptides, a finding consistent with that from mice supplied with exogenous LZ (58). The significant resistance to OS, usually associated with the delivery of exogenous AGE, may have been mediated by these two actions; however, the interplay between the reduction of serum AGE by cellular uptake and degradation of LZ:AGE complexes and rapid urinary excretion remains unknown. This, however, introduces a unique combination of properties of clinical interest. LZ is readily taken up by renal tubular cells and is either proteolytically cleaved by cathepsin B or excreted intact in the urine (20). The enhanced urinary excretion of AGE with increased levels of LZ suggests that the positively charged LZ molecule may favor an increased renal LZ:AGE excretion-to-retention ratio (58). Thus the new anti-AGE properties of endogenously expressed LZ found in the current study may be attributed to its antioxidant action, to its effect on renal AGE handling, or to both. In either case, LZ appears to act on OS induced by agents that can cause either acute or chronic OS stress. Thus, while deserving of further investigation, the underlying antioxidant mechanism(s) of LZ could be generalized to agents in addition to AGE. LZ action may be mediated by the formation of LZ complexes with AGE or other oxidants via its high-affinity, cysteine-bounded 18-amino acid ABCD motif (24), which attenuates OS, as well as via its own rapid renal elimination.

In summary, the in vivo overexpression of the native defense protein LZ can protect against acute OS, as well as confer resistance to chronic OS against milder oxidants, such as AGE. Thus structurally and functionally distinct oxidants may share aspects of intracellular mechanisms of injury via signaling pathways, involving key stress response genes, that are regulated by LZ and, possibly, other members of the family of defensins.

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REFERENCES


LYSOZYME AND OXIDANT STRESS


