Original Research

Human recombinant lysozyme downregulates advanced glycation endproduct-induced interleukin-6 production and release in an *in-vitro* model of human proximal tubular epithelial cells

Davide Gallo[1,5], Moreno Cocchietto[1], Elisa Masat[2], Chiara Agostinis[3], Elisa Harei[1], Paolo Veronesi[4] and Gianni Sava[2]

[1]Callerio Foundation Onlus, Institutes of Biological Researches, 34127 Trieste, Italy; [2]Department of Life Sciences, University of Trieste, 34127 Trieste, Italy; [3]Institute for Maternal and Child Health, IRCCS ''Burlo Garofolo'', 34137 Trieste, Italy; [4]Therapicon srl, 20146 Milano, Italy; [5]Department of Pharmacological Science, University of Padova 35 A 22, Padova, Italy
Corresponding author: Davide Gallo. Email: davide.gallo.3@studenti.unipd.it

Abstract

Diabetic nephropathy is the leading cause of chronic renal disease and one of the major causes of cardiovascular mortality. Evidence suggests that its progression is due to the chronic hyperglycemia consequent to the production and accumulation of advanced glycation endproducts (AGEs). Lysozyme was shown to posses AGE-sequestering properties and the capacity to reduce the severity of the early stage manifestations of the diabetic nephropathy. This study was aimed to contribute to the understanding the molecular mechanisms of lysozyme effectiveness in the diabetic nephropathy, using an *in-vitro* cellular model, represented by the HK-2 cells, human proximal tubular epithelial cells. Lysozyme significantly reduced the AGE-induced IL-6 mRNA and an ELISA assay showed also a decreased release of the functional protein with a dose-dependent trend. In addition, lysozyme prevented macrophage recruitment, suggesting its capacity to elicit an anti-inflammatory action. We may conclude that the protective action of lysozyme on the nephrotoxic effects of AGE may depend, at least in part, on its ability to prevent the production and release of inflammatory mediators, such as IL-6 and to reduce macrophage recruitment in the inflammatory sites.

Keywords: Lysozyme, advanced glycation endproducts, inflammation, diabetic nephropathy

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Introduction

Diabetic nephropathy is the most common cause of end-stage kidney disease in developed countries[1] with a significant impact on patient health and on the quality of life, also including the corresponding costs for patients care.[2,3] According to the results of the UK Prospective Diabetes Study (UKPDS) clinical research, chronic hyperglycemia, the typical condition in diabetes, is the most influent factor related to the development of nephropathic complications.[4,5] Diabetic nephropathy, similarly to other major complications of this disease, is considered to have a multifactorial origin. A growing body of evidences indicates that the development and progression of both microvascular and macrovascular diseases are strictly associated to the chronic hyperglycemia and to the consequent biochemical processes.[6] In fact, the main *trait d'union* between chronic hyperglycemia and diabetic nephropathy is the production of advanced glycation endproducts (AGEs).[6,7] AGEs are a group of modified proteins and/or lipids, formed by a non-enzymatic glycation and oxidation processes after the contact with aldose sugars,[8,9] an event known as Maillard reaction.[10] Early glycation and oxidation lead to the formation of Schiff bases and Amadori's products. The further glycation of proteins and lipids causes molecular rearrangements leading to the generation of AGE stable products, the formation of which is an irreversible event[11]; for an exhaustive explanation of AGE formation mechanisms and its relevance for diabetes, see Huebschmann et al..[12] AGEs tend to accumulate in the host tissues, altering their functions and the mechanical properties through the formation of cross-links with intracellular and extracellular matrix proteins.[13] AGE-induced effects are also due to their ability to interact, as specific ligands, with the membrane-bound receptor for advanced glycation endproducts (RAGE), isolated and characterized in 1992.[14] The AGE–RAGE interaction leads to a number of adverse phenomena, such as the generation of an excess of intracellular reactive oxygen species (ROS)[15–17] or to the enhanced transcription and production of a number of cytokines[18] and pro-inflammatory mediators

Table 1 Primer's sequences used for RT-PCR analysis

Sample	Primers	Sequence 5′ → 3′	Annealing temperature (°C)
18 S	Forward	ATCCCTGAAAAGTTCCAGCA	60°C
	Reverse	CCCTCTTGGTGAAGGTCAATG	
RAGE	Forward	GGGCAGTAGTAGGTGCTCAAA	61°C
	Reverse	CGGCCTGTGTTCCAGTTTCAT	
IL-6	Forward	GTACATCCTCGACGGCATC	60°C
	Reverse	CCAGGCAAGTCTCCTCATTG	
IL-18	Forward	TCTTCATTGACCAAGGAAATCGG	60°C
	Reverse	TCCGGGGTGCATTATCTCTAC	
CX3CL1	Forward	CCCAAAACTCTCCTCTGCTG	60°C
	Reverse	AGGTGCTCTGCTGGTAAG	
TNF-α	Forward	ATCAATGCCCCAGTCACC	60°C
	Reverse	CCCAAACTCCGAAGACT	

via the nuclear factor-κB (NF-κB) pathway,[19,20] e.g. the intercellular adhesion molecule-1 (ICAM-1), the vascular cell adhesion molecule (VCAM-1), E-selectin, the tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and cyclo-oxygenase-2 (COX-2).[21–23] The AGE–RAGE interaction can also induce other pathological events, such as increased production of the extracellular matrix[24] and the activation of autoimmune processes.[25]

Recent findings have shown lysozyme (LZ), the enzyme mainly known for its muramidase activity,[26] can beneficially act in the context of the diabetic nephropathy. LZ was shown to have a high binding affinity for AGE,[27] to be able to enhance AGE excretion *in vivo*[28] and to ameliorate AGE-induced oxidative stress.[29] We have also previously demonstrated that the orally administered microencapsulated LZ is able to reduce the severity of the diabetic nephropathy, on a preclinical model of streptozotocin-induced diabetic rats.[30]

The aim of the present work was therefore that of contributing to the comprehension of the molecular mechanisms involved in the protective action of LZ against AGE-induced cell damages, using the *in vitro* HK-2 cell model constituted by the human proximal tubule epithelial cells. In particular, the attention will be placed on the inflammatory events associated to the diabetic nephropathy, focusing on some pivotal pro-inflammatory mediators, such as the ROS and the related cytokines.

Materials and methods

Chemicals

Human recombinant lysozyme (Hr-LZ) was a gift of Dr P Veronesi, Therapicon srl, Milano.

Bovine serum albumin (BSA) fatty acid free, low endotoxin; D-glucose; insulin from bovine pancreas; holo-transferrin; T3 (3,3′,5-triiodo-L-thyronine); prostaglandin E_1 (PGE_1); sodium selenite; hydrocortisone; endothelial growth factor (EGF); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); isoamilic chloroform; 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP); 3,3′,5,5′-tetramethybenzidine (TMB); primer for reverse transcriptase polymerase chain reaction (RT-PCR) (Table 1); Phorbol 12-myristate 13-acetate (PMA/TPA); and RPMI 1640 were obtained from Sigma Aldrich® Chemical Co. (St Louis, MO).

Dulbecco's Modified Eagle's Medium (DMEM); Ham's F12; L-glutamine; penicillin and streptomycin; EuroGold Trifast™ were obtained from Euroclone® (Devon, UK).

Fetal bovine serum (FBS) was obtained from Gibco-Invitrogen™ (Paisley, Scotland, UK).

2′,7′-dihydrodichlorofluorescein-diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, OR).

iScript Reverse Transcriptase was obtained from Bio-Rad Laboratories (Hercules, CA).

Dynamo™ Flash SYBR® Green qPCR kit was obtained from Finnzymes, Vantaa, Finland.

AGE–BSA preparation

AGE–BSA was prepared by incubating BSA (50 mg/mL) at 37°C for six weeks with D-glucose 0.5 mol/L in a 0.2 mol/L phosphate buffer containing azide.[31] Then, preparation was extensively dialyzed against phosphate buffer to remove free glucose. The concentration of AGE, expressed in micromolar units, was determined considering the molecular weight of BSA used for the AGE preparation.

Cell culture

HK-2 cells, gift of Prof. R. Bulla (Department of Life Sciences, University of Trieste), an immortalized human proximal tubular epithelial cell line, were cultured and passaged in 25 cm^2 culture flasks that contained DMEM low glucose, Ham's F12 media (1:1) supplemented with decomplemented FBS 5%, antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin), L-glutamine 2 mmol/L, insulin from bovine pancreas 5 μg/mL, holo-transferrin 5 μg/mL, sodium selenite 5 ng/mL, hydrocortisone 5 ng/mL, EGF 10 ng/mL, T3 5 pg/mL, and PGE_1 5 pg/mL.

U937 cells, gift of Dr S Pacor (Department of Life Sciences, University of Trieste), a monocyte immortalized

cell line, were cultured and passaged in 25 cm^2 culture flasks that contained RPMI 1640 supplemented with FBS 10%, antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin), L-glutamine 2 mmol/L. All experiments were preceded by a 24-h period starvation, in a serum-free medium.

Viability assay

The determination of cell viability was measured by assessing the reduction of MTT to formazan by the mitochondrial enzyme, succinate dehydrogenase.[32,33] Cells were seeded in 96-well plates at the concentration of 5×10^3 cells/well, and maintained at 37°C in 5% CO_2 for 24 h, until confluence. AGE and LZ alone, and both together were then added in serum-free medium at the concentrations of 1, 10, and 20 μmol/L, for 24, 48, and 72 h. After the treatments, cells were incubated with MTT (5 mg/mL). After 4 h at 37°C, the supernatants were removed, the insoluble formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO) and the absorbance was determined at 570 nm using a spectrophotometer reader (SpectraCount™, Packard).

RNA isolation, cDNA synthesis, and RT-PCR

Cells were cultured in 24-well plates at the density of 5×10^4 cells/well. For RAGE investigation, cells were treated with LZ at the concentration of 1, 10, and 20 μmol/L for 24 and 96 h. For IL-6, IL-18, CX3CL1, and TNF-α investigation, cells were treated with AGE, LZ, and both at the concentrations of 1, 10, and 20 μmol/L for 24 h in serum-free medium. After the treatments, cells were harvested in EuroGold™ Trifast according to the supplier's instruction. Total RNA was extracted with chloroform and precipitated with isopropanol by 12,000 x *g* centrifugation at 4°C. In order to digest contaminant genomic DNA, RNA samples were treated with 5 U DNase free, re-extracted with omnizol/chloroform and precipitated with isopropanol. The RNA pellet was washed with 75% ethanol, resuspended in diethylpyrocarbonate-treated water. cDNA was synthesized from mRNA using iScript reverse transcriptase. Real-time quantitative PCR was carried out on a Rotor-Gene 6000 (Corbett, Explera, Ancona, Italy) using Dynamo™ Flash SYBR® Green qPCR kit.

Intracellular ROS detection

The quantification of ROS production was measured using the probe H_2DCFDA.[34] Cells were seeded in 96-well plates at the concentration of 5×10^3 cells/well, and maintained at 37°C in 5% CO_2 for 24 h, until the confluence. AGE were then added in serum-free medium at the concentrations of 10, 20, 30, and 50 μmol/L for short time (from 30 min to 6 h) and for longer time 24 h. At the end of treatments, the probe was added for 1 h at 37°C. Then, cells were lysed adding radioimmunoprecipitation assay (RIPA) buffer with Triton X-1%. The fluorescence was determined at 485 nm excitation and 530 nm emission, with a fluorescence reader (FluoroCount™ Packard).

Enzyme-linked immunosorbent assay (ELISA)

IL-6 released in the supernatants was quantified by means of a sandwich ELISA assay. Firstly, cells were seeded in 96-well plates at density of 5×10^3 cells/wells and maintained at 37°C for 24 h, until confluence. Then, cells were treated with AGE, LZ, and both at the concentrations of 1, 10, and 20 μmol/L for 24 h in a serum-free medium. After 24 h of treatments, supernatants were picked up and stored at −80°C, until the use. For ELISA assay, a Peprotech Human IL-6 kit was used. Briefly, 96-well plate was coated with capture antibody with a final concentration of 100 μg/mL, at room temperature overnight. Then, after four washes, block buffer was added in order to saturate the unspecific sites. After 1 h at room temperature, standard and samples were incubated at room temperature for 2 h. Subsequently, a detection antibody at the concentration of 0.25 μg/mL was incubated for 2 h at room temperature. After this incubation, the secondary antibody, avidin-horseradish peroxidase (HRP) conjugated, was used, diluted 1:2000, for 30 min at room temperature. The final step was to add TMB, the substrate of HRP, and to monitor the color development. To block the reaction 2 N sulfuric acid was used. The plate was read at 450 nm by means of a spectrophotometer reader (PowerWave, X Bio-Tek Instruments, Ink).

Migration assay

The ability of LZ to influence the AGE-induced U937 migration was assessed by a migration assay. In order to induce monocytic differentiation, U937 cells were first activated with PMA/TPA 50 ng/mL for 72 h. Cell migration was assayed in a 24-well plate, using Transwell® insert characterized by a polycarbonate filter with 8 μm pores. U937 cells at the concentration of 1.5×10^5, resuspended in 100 μL, were seeded in the inserts while in the lower chamber were placed supernatants obtained treating HK-2 cells with 1, 10, and 20 μmol/L of AGE and LZ. The assembled migration plate chamber system was incubated at 37°C for 90 min.

In order to determine the amount of migrated cells, the cells attached to the upper parts of the polycarbonate filters were removed, while the cells attached on the lower parts of the filters and in the bottom chambers were fixed by means of glutaraldehyde 1.1% for 15 min. Then, the cells were washed with distilled water and stained with crystal violet 0.1% in borate buffer 200 mmol/L, pH 9, for 20 min. After other three washes with distillated water, the excess of dye was removed. Crystal violet that stained cells was eventually solved through acetic acid 10% v/v for 10 min. The data were acquired by means of a spectrophotometer reader (SpectraCount™Packard) at a wavelength of 570 nm.

Statistical analysis

Experimental data were subjected to computer-assisted ANOVA statistical analysis using Tukey-Kramer post test (Instat 2, GraphPad Software, San Diego, CA). Differences of $P < 0.05$ were considered to be significant.

Results

The concentrations of AGE used in the present study were taken from literature data[35,36] and from previous studies performed in our laboratories.

Cytotoxicity of AGE

The effects of AGE on the viability of HK-2 cells are reported in Figure 1. Treatment for 24 h at concentrations of 10 and 20 μmol/L AGE reduced the viability of HK-2 in a statistically significant and dose-dependent way, respectively, by 20% and 50% (Figure 1a). Cell challenges with AGE of 48 and 72 h produced similar result without any further increase of cytotoxicity over that observed after 24 h (Figure 1b and c).

LZ is completely free of effects on the viability of HK-2 cells at 20 μmol/L concentration and 72 h treatment. Also, LZ, at 1–20 μm l/L concentrations, is unable to prevent or reduce the cytotoxicity induced by AGE (unreported results). The doses of LZ used were selected in order to get a 1:1 ratio with the concentration of AGE used.

RAGE mRNA quantification

The expression levels of RAGE in HK-2 cells, following exposure to LZ, as determined by RT-PCR showed no variation in the levels of mRNA for RAGE when HK-2 cells were exposed to 1–20 μmol/L LZ for 24–96 h, independently of the dose and of the length of cell exposure to this compound. Comparison is made versus cells in their own medium (Figure 2a and b).

Intracellular ROS detection

The quantification of intracellular ROS production by HK-2 cells after treatments with 10–50 μmol/L AGE is reported in Figure 3. Unlike the positive standard (ABAP), neither after short treatments (at 30 min to 6 h cell exposure to AGE) (Figure 3a) nor after longer treatments for 24 h (Figure 3b) these cells showed increased ROS levels, at any of the concentrations used.

IL-6, IL-18, CX3CL1, and TNF-α mRNA quantification

Unlike ROS induction, data presented in Figure 4 show AGE to induce a significant increase of the production of IL-6. AGE at concentration of 1–20 μmol/L increased the mRNA levels of IL-6, measured by means RT-PCR, from 30% to 70% versus the controls. In the same experimental conditions, 1–20 μmol/L LZ, as expected, were devoid of any effect on IL-6 production, whereas it decreased the

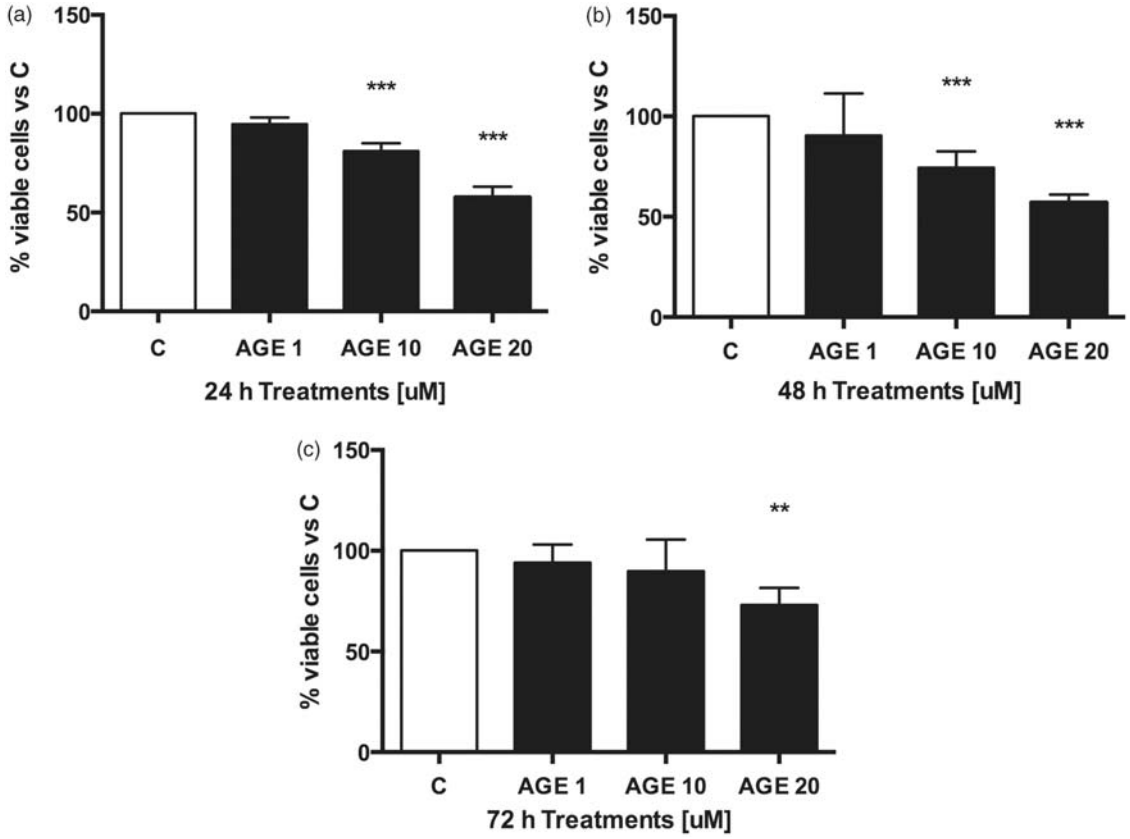

Figure 1 Effects of 1, 10, and 20 μmol/L AGE treatments on cells viability evaluated by means of MTT test. a: Cells incubated with AGE for 24 h. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. ***$P < 0.001$ versus C. b: Cells incubated with AGE for 48 h. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. ***$P < 0.001$ versus C. c: Cells incubated with AGE for 72 h. Data are expresses as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. **$P < 0.01$ versus C. AGE: advanced glycation endproduct; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

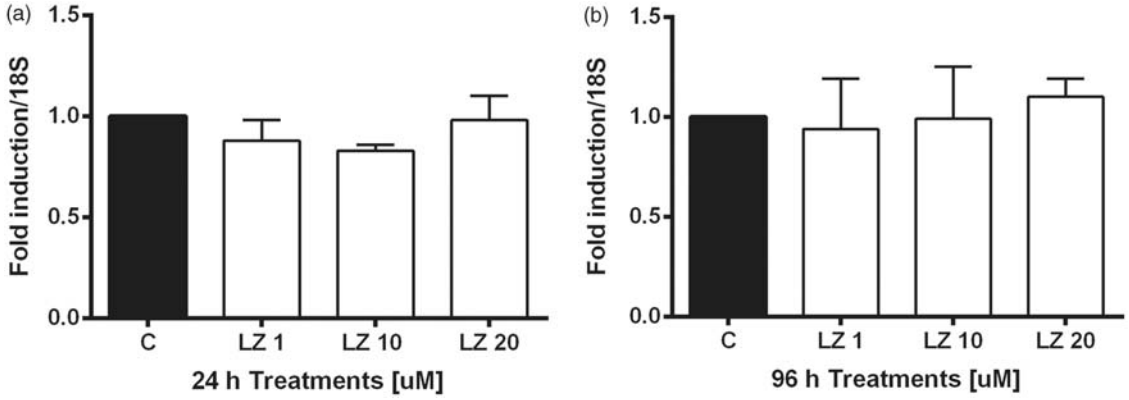

Figure 2 Effects of 1, 10, and 20 μmol/L LZ-treatment on RAGE mRNA levels by means of RT-PCR. a: Cells incubated with LZ for 24 h show no significant variations versus C. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. b: Cells incubated with LZ for 96 h show no significant variations versus C. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. LZ: lysozyme; RAGE: receptor for advanced glycation endproducts; RT-PCR: reverse transcriptase polymerase chain reaction

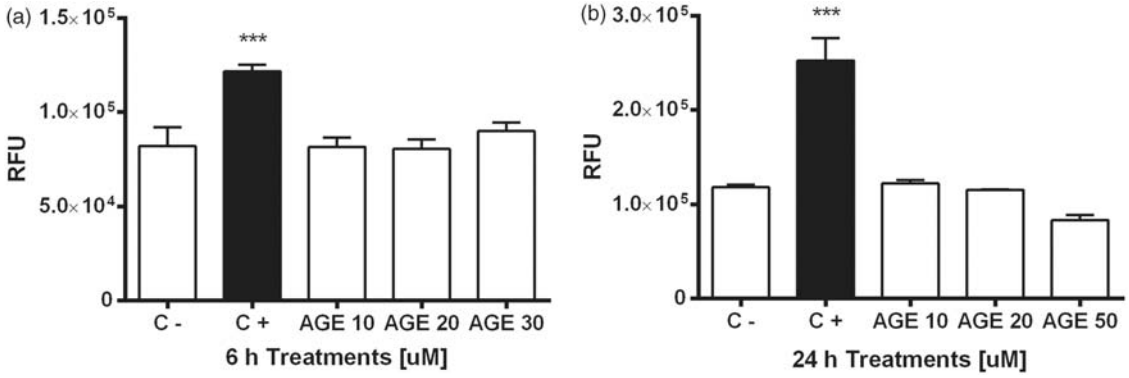

Figure 3 Quantification of intracellular ROS by means H_2DCFDA probe. Positive controls (C+): cells treated with ABAP 1 mmol/L. Negative controls (C−): cells in own medium. a: 10, 20, and 30 μmol/L AGE treatments for 6 h of AGE. No significant variations were measured between treatments and negative control. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. ***$P < 0.001$ versus C−. b: 10, 20, and 50 μmol/L AGE-treatments for 24 h of AGE. No significant variations were measured between treatments and negative control. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. ***$P < 0.001$ versus C−. ABAP: 2,2'-azobis (2-methylpropionamidine) dihydrochloride; AGE: advanced glycation endproduct; ROS: reactive oxygen species

AGE-induced IL-6 production to lower values, with a dose-dependent relationship.

Concerning the quantification of mRNA for the other cytokines tested, namely IL-18, CX3CL1, and TNF-α, data reported, respectively, in Figure 5, panel A, B and C, show no significant modifications versus controls after 1–20 μmol/L AGE treatments. In these conditions, LZ alone globally decreases the levels of IL-18 and CX3CL1 mRNA, but has no effects on TNF-α. The effects of LZ on the mRNA expression of IL-1, MCP-1, and RANTES, with or without AGE, are not reported, considering that the levels of mRNA measured in these experiments were very low in the basal conditions (controls).

IL-6 ELISA assay

LZ at concentration of 1–20 μmol/L also reduced the release of IL-6 stimulated by 1–20 μmol/L AGE in HK-2 cells. AGE induced a dose-dependent increase of the release of IL-6 (from 2-fold at 1 μmol/L to 5-fold at 20 μmol/L) (Figure 6); the concomitant use of LZ significantly reduced the AGE-induced release of IL-6, and this reduction was significantly greater at the higher concentrations of LZ used (from −30% at 1 μmol/L to the complete prevention at 20 μmol/L).

Migration assay

Data reported in Figure 7 show that the use of supernatants taken from HK-2 cells treated for 24 h with 1, 10, and 20 μmol/L AGE caused a significant increase of the migration ability of the tested U937 macrophages (from 2-fold at 1 μmol/L to 3-fold at 20 μmol/L). LZ was unable to modify the migratory activity of U937 macrophages, whereas when given to HK-2 cells, in the presence of AGE, it was able to statistically reduce the migration-induction effect of the supernatants of these cells on U937 macrophages (from 25% at 1 μmol/L to 50% at 20 μmol/L).

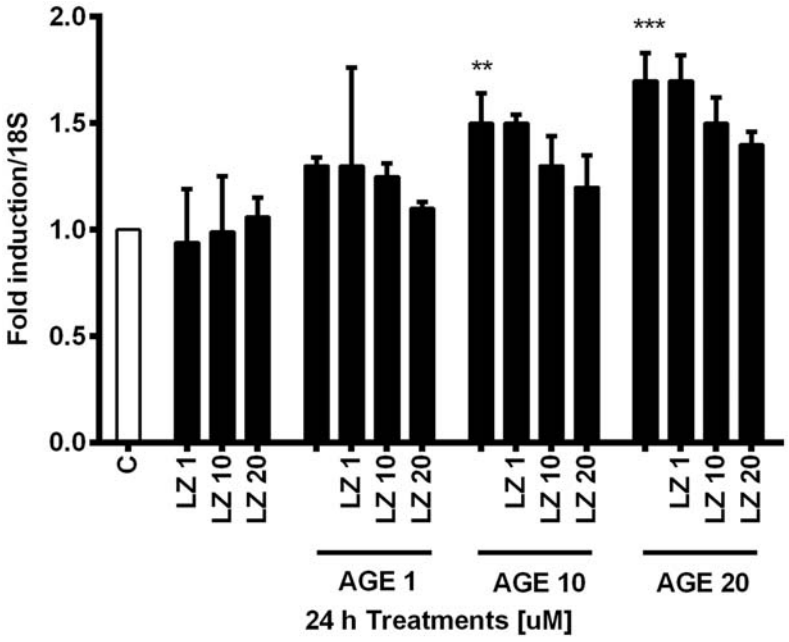

Figure 4 IL-6 mRNA levels quantification by means of RT-PCR. AGE-treatment at concentrations of 1, 10, and 20 μmol/L for 24 h increase significantly IL-6 mRNA levels. LZ at concentrations of 1, 10, and 20 μmol/L do not influence IL-6 mRNA levels. Contemporary treatment with LZ and AGE restore IL-6 mRNA levels to value comparable to the controls. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. ***$P < 0.001$ versus C; **$P < 0.01$ versus C. AGE: advanced glycation endproduct; IL: interleukin; LZ: lysozyme; ROS: reactive oxygen species; RT-PCR: reverse transcriptase polymerase chain reaction

Discussion

Diabetic nephropathy, one of the major diabetic complications, is the leading cause of end-stage kidney disease in Western countries. A number of evidences link diabetic complications to the great increase of formation and accumulation of AGE occurring in diabetic patients. LZ can play a role in the diabetic nephropathy by acting as an AGE' scavenger[28] and/or preventing some of the early manifestations of the diabetic nephropathy, such as microalbuminuria and glomerular hypertrophy.[30]

AGE interactions with their receptors can induce a number of events, including the enhanced oxidative stress[6] and AGE were also shown to be involved in other inflammatory phenomena.[20] In the present work, we show LZ to modulate important events associated to inflammatory processes such as macrophages mobility. Importantly, these results were obtained in a cell line (HK-2) mimicking the proximal tubule of the kidney, the site where diabetic hyperglycemia exerts a large part of its pathological changes.

It must be highlighted that the cellular model used in this work was not able to show an AGE-induced increased oxidative stress. In fact, also using different approaches and probes, such as H_2DCFCDA and DHE (data not reported), known to be able to detect a wide range of ROS (nitric oxide, peroxynitrite anions, organic hydroperoxides, superoxide anions)[37–42] in a number of conditions (low and high concentrations after short and prolonged treatments), no significant variations were detected. The lack of AGE-induced increase of ROS might depend on the difficulty to simulate *in-vitro* events that are quantifiable *in vivo* after prolonged exposure to the stimulating agents. It is remarkable to note that cells cultured *in vitro* have lost their capacity to regulate the RAGE expression. This effect might explain the lack of the AGE-induced RAGE upregulation (data not reported) and the consequent LZ downregulation, as it was expected from the data reported after an *in-vivo* study.[30] Considering that ROS production is strictly associated to the AGE-RAGE modulation, the lack of activation of this axis might explain the absence of the expected increase of ROS production in our conditions.

However, according to the available literature, LZ activity in inflammation can be related also to a number of other events, among which we can include the induction of macrophage migration and the consequent related cascade of immunological events. In fact, LZ interferes with the AGE-induced macrophage recruitment, and macrophages are one of the central mediators of the renal vascular inflammation; their accumulation in the renal tissue is a characteristic feature of the diabetic nephropathy.[43–46]

There are several soluble factors involved in macrophages migration in the context of inflammation. We focused our attention on some chemokines and cytokines involved in the pathological process of the diabetic nephropathy, such as MCP-1, RANTES, IL-1, CX3CL1, TNF-α, IL-6, and IL-18.[20] Here we report the results on IL-6, IL-18, CX3CL1, and TNF-α while the results on MCP-1, RANTES, and IL-1 are not illustrated in detail. It is interesting to note that in our experimental conditions, with the exception of IL-6, none of the chemokines (MCP-1, CX3CL1, RANTES), and none of the other cytokines tested (TNF-α, IL-1, IL-18), are upregulated by AGE.

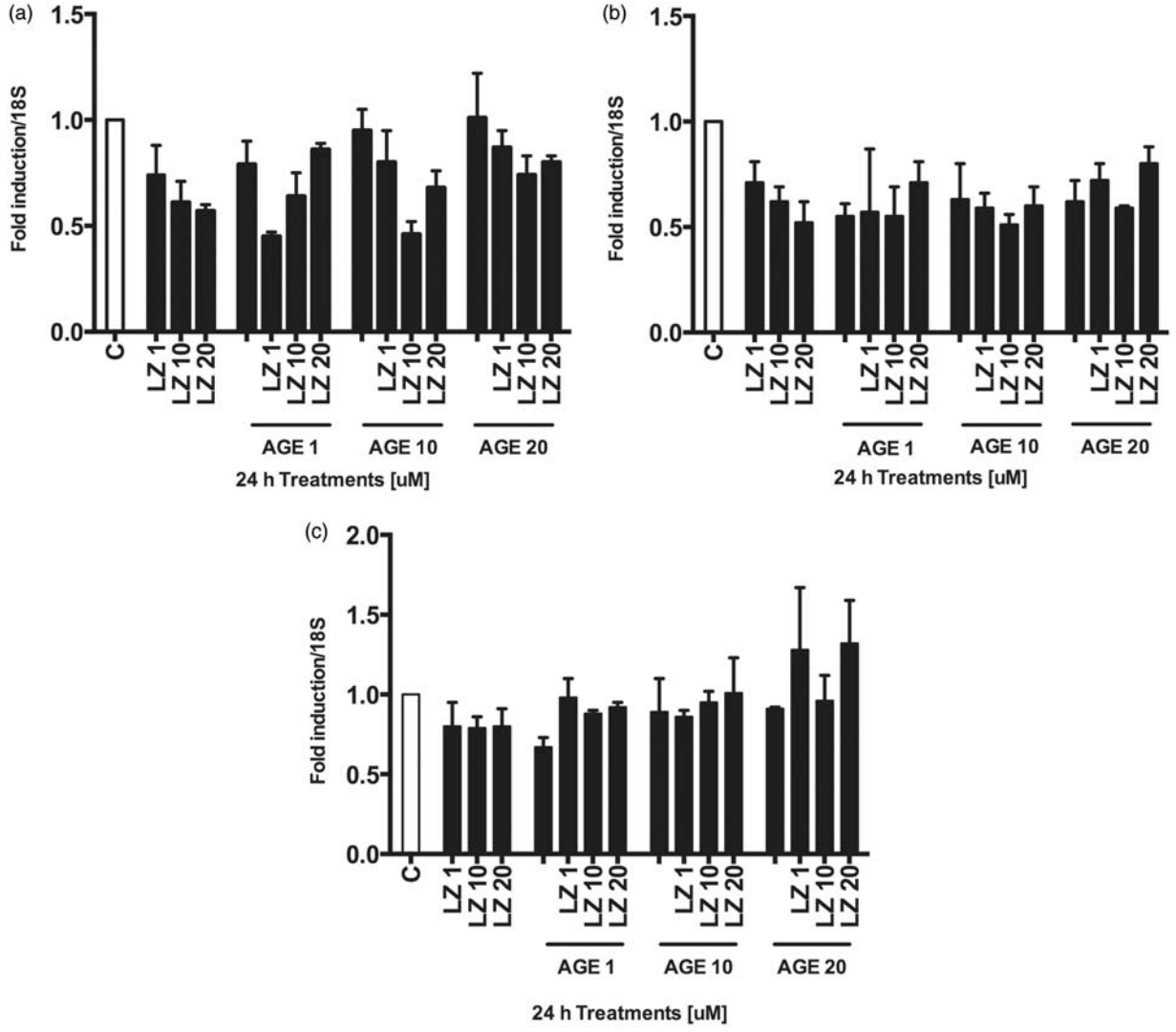

Figure 5 a: IL-18 mRNA levels quantification by means of RT-PCR. 1, 10, and 20 μmol/L AGE and LZ treatments induce no significant variations versus C. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. b: CX3CL1 mRNA levels quantification by means RT-PCR. 1, 10, and 20 μmol/L AGE and LZ treatments induce no significant variations versus C. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. c: TNF-α mRNA levels quantification by means RT-PCR. AGE at the concentrations of 1, 10, and 20 μmol/L and LZ treatments induce no significant variations versus C. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. AGE: advanced glycation endproduct; IL: interleukin; LZ: lysozyme; ROS: reactive oxygen species; RT-PCR: reverse transcriptase polymerase chain reaction; TNF: tumor necrosis factor

Globally, also LZ has no significant effects on these inflammatory factors when they are not modified by AGE. Concerning IL-6 release and production, it is interesting to note that LZ was unable to modulate the production of this cytokine in intact healthy HK-2 cells but it was capable to inhibit that elicited by AGE, in terms of mRNA levels and in comparison to untreated controls. These results were also confirmed by ELISA assays of the protein showing the capacity of LZ to inhibit the release of IL-6 in the supernatants of the treated cells in a dose-dependent manner.

The modulation of IL-6 production and release is considered a pivotal event in the development of diabetic nephropathy. In fact, cells infiltrating the mesangium, interstitium, and tubules were shown positive to mRNA encoding IL-6.[47] These data were confirmed by another, more recent, research where IL-6 was shown to be significantly overexpressed in diabetic rat kidneys, with increased levels of mRNA encoding IL-6 in the renal cortex directly associated with the increase in its urinary excretion.[48] In addition, a study by Seizuka et al.[49] showed that serum levels of IL-6, in patients with diabetic nephropathy, were significantly higher than in diabetic patients without kidney injury.

Concerning the effects of LZ in the inflammatory response, a large amount of aspects are still unknown. Nevertheless, a work by Gordon et al.,[50] demonstrated LZ's to modulate chemotaxis of polymorphonuclear (PMN) cells *in vitro,* although the mechanisms was not clear. LZ also showed the ability to inhibit the superoxide generation, not quenching the already formed superoxide anion but, probably, acting through a membrane-dependent function.

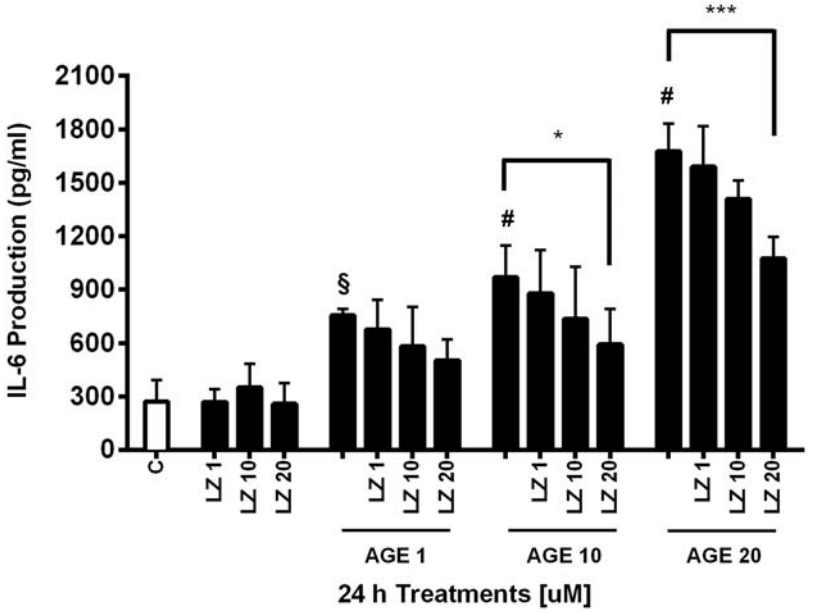

Figure 6 ELISA assay to evaluate the effects of LZ on the release of IL-6. IL-6 detection after treatments with 1, 10, and 20 μmol/L of AGE show a significant dose-dependent increase in IL-6 release. LZ at concentrations of 1, 10, and 20 μmol/L do not influence IL-6 release. Contemporary treatments with LZ reduce significantly the AGE-induced increase in IL-6 release. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. § = $^{**}P < 0.01$ versus C; # = $^{***}P < 0.001$ versus C; a = $^{***}P < 0.001$ versus AGE 1 and AGE 10; $^{*}P < 0.05$ versus AGE 10 + LZ 20; $^{***}P < 0.001$ versus AGE 20 + LZ 20. AGE: advanced glycation endproduct; ELISA: enzyme-linked immunosorbent assay; IL: interleukin; LZ: lysozyme

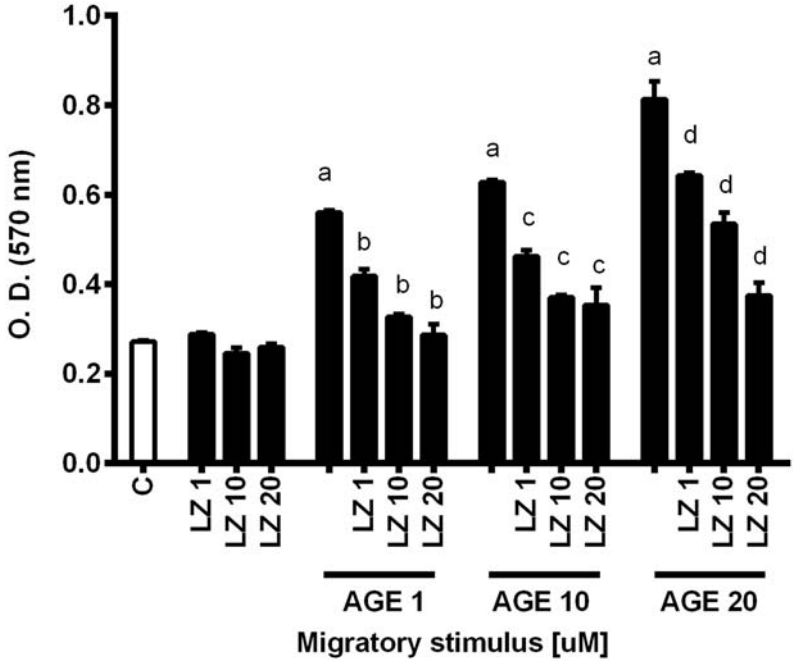

Figure 7 Migration assay. U937 cells migrated after 90 min treatments with supernatants of HK-2 cells 1, 10, and 20 μmol/L AGE-stimulated, 1, 10, and 20 μmol/L LZ-stimulated and contemporary treatments with LZ and AGE stimulated. Data are expressed as means ± SD. Statistical analysis: ANOVA followed by Tukey-Kramer. a = $^{***}P < 0.001$ versus C; b = $^{***}P < 0.01$ versus AGE 1; c = $^{***}P < 0.001$ versus AGE 10; d = $^{***}P < 0.001$ versus AGE 20. AGE: advanced glycation endproduct; LZ: lysozyme

Rather than to an effect on the AGE-RAGE axis, LZ-mediated IL-6 reduction could be attributable to some ''alternative'' mechanism. For example, to the capacity of LZ to inhibit the AGE-activated NF-κB axis, through p38 phosphorylation, as resulting from a preliminary result (Callerio Foundation, data on file, 2013). In addition, a number of data suggest that the AGE–LZ interaction could determine the increase of the lysosome degradation of the complex.

Taken together, these data open the way to study the effects of LZ on the cell pathways involved in the elicitations of the inflammatory processes, such as p38 MAPK

and/or NF-κB, and particularly on the target with which LZ interacts to produce the anti-inflammatory effects observed in the HK-2 cell model.

In conclusion, the present study indicates that the possible molecular mechanism of action of LZ responsible for its AGE-protecting action may be related to its anti-inflammatory activity. In fact, in our *in-vitro* model, LZ shows the ability to reduce the production and release of a typical inflammatory mediator, such as IL-6 and to reduce another pivotal manifestation of inflammation, such as macrophage recruitment. Among all, these results might open the way to the use of LZ, as a safe, simple, economic, and effective drug, suitable for the oral use, for the control of the progression of the diabetic nephropathy.

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