Protein Disulfide Levels and Lens Elasticity Modulation: Applications for Presbyopia

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Submitted: October 14, 2015
Accepted: March 21, 2016

Citation: Garner WH, Garner MH. Protein disulfide levels and lens elasticity modulation: applications for presbyopia. *Invest Ophthalmol Vis Sci*. 2016;57:2851–2863. DOI:10.1167/iovs.15-18413

Purpose. The purpose of the experiments described here was to determine the effects of lipoic acid (LA)-dependent disulfide reduction on mouse lens elasticity, to synthesize the choline ester of LA (LACE), and to characterize the effects of topical ocular doses of LACE on mouse lens elasticity.

Methods. Eight-month-old mouse lenses (C57BL/6J) were incubated for 12 hours in medium supplemented with selected levels (0–500 μM) of LA. Lens elasticity was measured using the coverslip method. After the elasticity measurements, P-SH and PSSP levels were determined in homogenates by differential alkylation before and after alkylation. Choline ester of LA was synthesized and characterized by mass spectrometry and HPLC. Eight-month-old C57BL/6J mice were treated with 2.5 μL of a formulation of 5% LACE three times per day at 8-hour intervals in the right eye (OD) for 5 weeks. After the final treatment, lenses were removed and placed in a cuvette containing buffer. Elasticity was determined with a computer-controlled instrument that provided Z-stage upward movements in 1-μm increments with concomitant force measurements with a Harvard Apparatus F10 isometric force transducer. The elasticity of lenses from 8-week-old C57BL/6J mice was determined for comparison.

Results. Lipoic acid treatment led to a concentration-dependent decrease in lens protein disulfides concurrent with an increase in lens elasticity. The structure and purity of newly synthesized LACE was confirmed. Aqueous humor concentrations of LA were higher in eyes of mice following topical ocular treatment with LACE than in mice following topical ocular treatment with LA. The lenses of the treated eyes of the old mice were more elastic than the lenses of untreated eyes (i.e., the relative force required for similar Z displacements was higher in the lenses of untreated eyes). In most instances, the lenses of the treated eyes were even more elastic than the lenses of the 8-week-old mice.

Conclusions. As the elasticity of the human lens decreases with age, humans lose the ability to accommodate. The results, briefly described in this abstract, suggest a topical ocular treatment to increase lens elasticity through reduction of disulfides to restore accommodative amplitude.

Keywords: presbyopia, treatment, eye-drops

Accommodation involves subtle movements of structures within the eye. The changes in lens shape,[1–3] responsible for accommodation, are governed by the ciliary muscle through ligaments called zonules (the zonules of Zinn, anteriorly; the vitreous zonules, posteriorly).[4–6] The parasympathetic innervation originating in the Edinger-Westphal nucleus carried by oculomotor nerve (cranial nerve 3) through the ciliary ganglion regulates the ciliary muscle.[7–9] Although there are aging changes in each of these components of accommodation, age-related changes in the lens correlate best with the age-dependent decrease in accommodative amplitude.[10–14]

Although primary fibers are formed in the embryonic period, additional new shells of secondary fibers are added synchronously throughout life. As these new shells of fibers differentiate, they elongate, lose anterior apical-apical epithelial-fiber attachments and posterior basal-capsular attachments, and form the anterior and posterior sutures.[15,16] There is a programmed loss of cellular organelles during fiber differentiation.[17]

The opposite end curvature of fibers at the anterior and posterior sutures make primate lens fibers into coils or springs that respond to capsular tension.[18] Accommodation is accomplished via zonular tension differences on the capsule. In the unaccommodated state (ciliary muscle relaxed), taut zonules of Zinn increase lens capsular tension causing the fibers to abut one another end to end at the anterior and posterior sutures. This causes a decrease in lens thickness and an increase in equatorial diameter. In the accommodated state (ciliary muscle contracted), lax zonules of Zinn decrease capsular tension, causing an increase in lens thickness and decrease in equatorial diameter. The fiber ends in the accommodated state interface with one another at the suture rather than abut end to end.

However, over a lifetime, changes in lens shape and elasticity occur due to the continuous production of lens secondary fibers, and the fiber spring mechanism becomes less efficient. First, although the anterior-to-posterior thickness is constant, the ratio of sagittal thickness to equatorial diameter increases with age.[10,19] Ideally, the anterior surface flattens and

radius of curvature increases, but posterior radius of curvature does not significantly increase or decrease with age. These changes in lens shape and size correlate with the age-dependent decrease in accommodation. As the lens grows, it becomes less elastic, especially the lens nucleus.[20–22] The increasingly larger and less elastic lens is sufficient to account for the loss of accommodative amplitude up to the age of 55 years.[10,15,20,23]

A significant age-dependent increase in lens protein disulfides has been observed in human lenses. However, to date, no experimental evidence links increased protein disulfides to increased lens stiffness. In human lenses, glutathione (GSH) levels correlate negatively with age in both the lens cortex and nucleus.[24,25] There is an age-dependent formation of mixed disulfides between protein sulfhydryl groups (PSH) and GSH's sulfhydryl group (PSSG) and between PSH and cysteine (PSSC).[25] Several crystallins of the β/γ family are glutathionylated increasingly with age.[26,27] There is an age-dependent increase in intramolecular protein disulfides in αA crystallin.[28] Even at the young age of 5 years, αA crystallin, as well as crystallins of the β/γ family, are involved in both intramolecular and intermolecular protein disulfides.[29] Although in the young human lens the intramolecular disulfides predominate, by the age of 67 years the intermolecular protein disulfides predominate.[29]

Based on the age-dependent increases in lens stiffness and lens disulfides, might treatment of the lens with an exogenous antioxidant increase lens elasticity? Because both cortical and nuclear fibers have GSH reductase (GR) and thioredoxin reductase (TR),[30] R-lipoic acid (LA; International Union of Pure and Applied Chemistry Identification: [R]-5-[1,2-dithiolan-3-yl]pentanoic acid) was chosen to answer this question.

R-Lipoic acid is an oxidized disulfide whose reduction by GR[31,32] or TR[33] produces the potent antioxidant, dihydrolipoic acid (DHLA). The antioxidant activities of DHLA include scavenging reactive oxygen and nitrogen species, regeneration of cellular antioxidants like GSH and cysteine, and chelation of heavy metals.[34–37] Because oxidative stress is one of the factors associated with insulin resistance and type 2 diabetes,[38] DHLA, through LA, ameliorates some of the symptoms or complications of the disease. Systemic administration of LA (600 mg/d) for 3 weeks or oral administration of LA (600 mg/d) for 5 weeks safely and effectively reduces the pain from diabetic sensorimotor polyneuropathy.[39–42] Lipoic acid is also an endogenous cofactor. In mitochondria of eukaryotes, LA is synthesized from the precursor octanoic acid and is covalently bound through an amide linkage to specific lysine residues of the pyruvate dehydrogenase complex, the α-ketoglutarate dehydrogenase complex, and the branched-chain oxoacid dehydrogenase complex, where its job as a shuttle is essential for aerobic metabolism.[43–47]

Mice and their lenses were used to demonstrate that treatment with LA and its choline ester (LACE) reduce protein disulfides and make the lens more elastic. Although there is no evidence for accommodation in the mouse eye, the mouse lens shares many similarities to the human lens. First, protein disulfides increase with age.[48] Like the lenses of young rats,[49] lenses of young mice have both intramolecular protein disulfides and intermolecular protein disulfides.[29,49] Similar to the lenses of a 5-year-old human, intramolecular disulfides predominate in young mouse and rat lenses.[29,49] Furthermore, old mice or rats with clear lenses have mostly intermolecular protein disulfides, as do lenses of aging humans.[29,49] Second, there is an age-dependent increase in the stiffness of the mouse lens.[50,51] Third, although the anterior curvature of the lens increases with age, the age-dependent change in the posterior lens curvature is unremarkable.[52] Finally, the lens nucleus is less elastic than the lens cortex.[51] Because of these similarities, mice and their lenses were used to demonstrate that antioxidants with a 1,2-dithiolane ring reduce protein disulfides and make the lens more elastic. The results of these studies are presented in this report.

Methods

Animals

The ARVO Statement for the Use of Animals in Research was adhered to in these studies. Eight-week-old (young) and 8-month-old (old) mice, strain C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA) were housed singly or in groups of two in cages in a room, 21° to 24°C, with 12/12 light-dark cycles. They were supplied with standard mouse chow and water ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee, University of North Texas Health Science Center, Fort Worth, TX, USA.

Antioxidants

We obtained (R)-5-(1,2-dithiolan-3-yl)pentanoic acid (R-lipoic acid or R-thioctic acid, LA) from Sigma-Aldrich Corp. (St. Louis, MO, USA). The iodide salt of the choline ester of (R)-5-(1,2-dithiolan-3-yl)pentanoic acid (lipoic acid choline ester, LACE), a proprietary compound of Encore Vision (Fort Worth, TX, USA), was synthesized using the procedure outlined in Patent number US 20100317725.[53]

Lens Culture (In Vitro) Experiments

Old mice were euthanized by CO_2 overdose on the day of the experiment. Their eyes were enucleated and rinsed with Hank's balanced salt solution (HBSS) with calcium and magnesium (Invitrogen, Carlsbad, CA, USA) supplemented with 1 mL/100 mL antibiotic-antimycotic solution (×100; Invitrogen).

Lenses were removed from each mouse eye and placed in individual wells of 24-well culture plates in culture medium 200 (Invitrogen), supplemented with antibiotic-antimycotic solution (×100) (Invitrogen) (1 mL/100 mL medium). The freshly dissected lenses were incubated for 2 hours at 37°C in an atmosphere of 5% CO_2/95% air to recover metabolite levels. Then, the medium was replaced with fresh medium supplemented with selected levels of LA (experimental lenses) or with fresh medium (contralateral control lenses). Lipoic acid levels ranged from 1 μM to 500 μM. After a 12-hour incubation at 37°C in an atmosphere of 5% CO_2/95% air, lenses were removed from culture medium, rinsed three times with a sterile balanced salt solution (HBSS with calcium and magnesium; Invitrogen), weighed, and analyzed for changes in elasticity and disulfide content.

Elasticity Measurements for In Vitro Experiments

For the lens culture experiments, modification of the coverslip lens-squeezing method[51] was used (Fig. 1, left, top). Each lens was placed on a slide on a grid (50 μm/division) and photographed using either a Retiga Exi (QImaging, Surrey, BC, Canada) camera with ×1 or ×4 objective of the Nikon (Melville, NY, USA) TE200-U microscope, or an Olympus (Center Valley, PA, USA) E330 camera with the ×1 objective of the Olympus dissection microscope (Fig. 1b). A second photograph was taken after a coverslip (exerting a force of ~100 mg) was placed on the lens (Fig. 1c). A photograph of the 50-μm grid was also obtained (Fig. 1a). The photographs were saved as TIFF files for later analysis. Composite contact prints of the photographs for each LA concentration of 1 μM, 5

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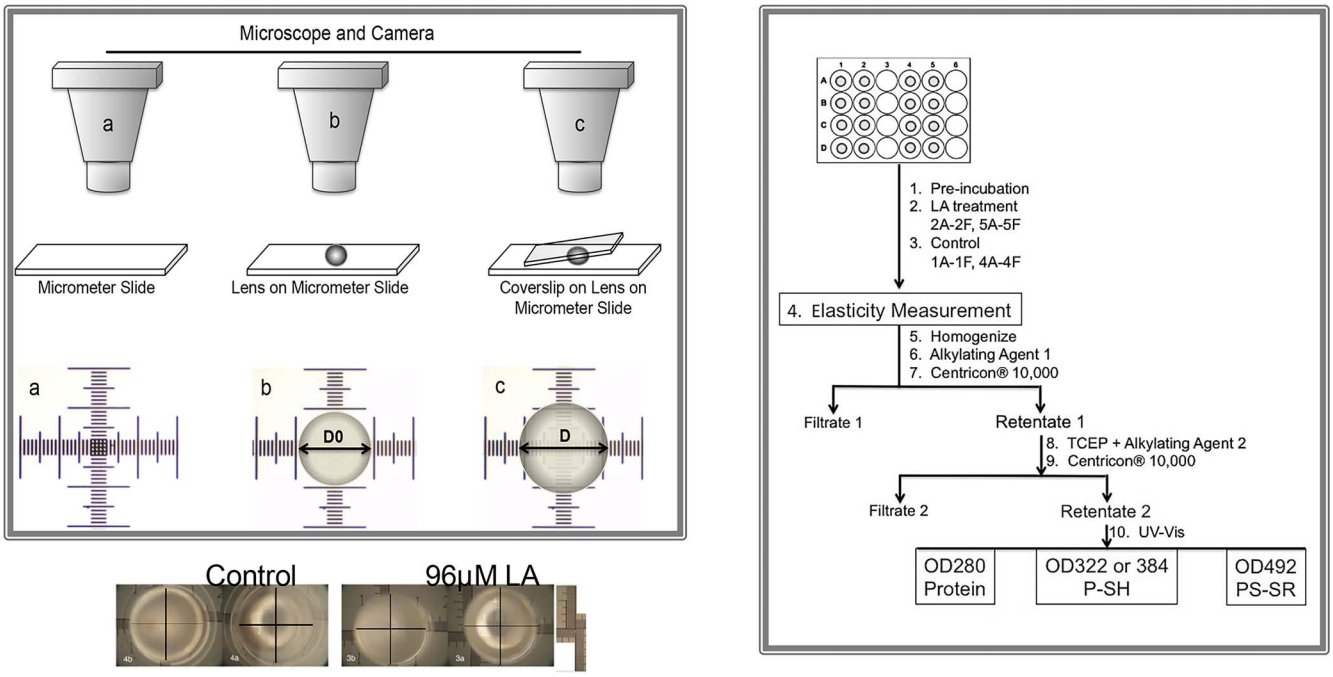


Figure 1. Flow charts for methods used to determine lens elasticity (*left*) and lens protein disulfide levels (*right*) for lens culture experiments.

μM, 9.6 μM, 50 μM, 96 μM, and 500 μM were prepared in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). Equatorial diameters were validated using the micrometer scale in the photo (one diameter per photo). The diameters were first measured as pixel distances using Adobe Photoshop. The pixel diameters were then converted to micrometers by dividing the diameter in pixels by the number of pixels per 1000 μm (one to two diameters/photo) using the external scale. Figure 1, left, bottom, shows a representative lens pair: a control lens (OS) and the experimental lens (OD) treated with 96 μM LA before and after coverslip squeezing. Averages of the two to three measurements were calculated and recorded in column format in a PRISM file (PRISM6 for Mac OS 10; GraphPad Software, Inc., La Jolla, CA, USA) for statistical analysis.

Protein Disulfide Bond Measurements

Once the lenses were photographed, they were placed in individual 2-mL conical bottom microfuge tubes in 400 μL homogenization medium (HMS) that contained 50 mM HEPES buffer, pH 8.4, and 0.1% SDS (Fig. 1, right, shows lens protein disulfide methods flow chart). Each lens was homogenized at room temperature in its microfuge tube with a conical pestle. Because of the presence of the denaturant, SDS, in the HMS, there was no insoluble protein except the capsule. Sufficient alkylating agent 1 to make a final concentration of 5 ± 0.2 μM was added to each homogenate. Alkylating agent 1 was 4-acetamido-4′-maleimidylstilbene (ST322 with an absorption maximum 322 nm and an extinction coefficient of 35,000, Molecular Probes, Life Technologies, Eugene, OR, USA) or 7-diethylamino-3-(4′-maleimidylphenyl)-4-methyl-coumarin (CPM384 with an absorption maximum at 384 nm and an extinction coefficient of 45,000; Molecular Probes, Life Technologies). After reaction in the dark for 2 hours at 4°C (ST322) or at room temperature (CPM384), the protein was separated from the reactant using Centricon filtration units (10,000 nominal molecular weight cutoff; Millipore, Billerica, MA, USA). The filtrate that contained low molecular weight sulfhydryl groups (nominal molecular weight <10,000) like reduced and oxidized GSH as well as other low molecular weight metabolites was discarded. The retentate (protein) was rinsed with 400 μL fresh HMS using the filtration units until no ST322 or CPM384 was found in the filtrate. Each retentate was diluted to 400 μL with HMS that contained 2.86 mg/mL of the reducing agent, tris(2-carboxyethyl)phosphine (reducing agent TCEP; Sigma-Aldrich Corp.) before being placed in new microfuge tubes. Sufficient alkylating agent 2, to make a final concentration of 5.0 ± 0.2 μM was added to each tube. Alkylating agent 2 was fluorescein –5-maleimide (FL492 with an absorption maximum at 515 nm and an extinction coefficient of 83,000; Molecular Probes, Life Technologies). The reaction mixtures were incubated in the dark for 3 hours at 37°C. Then the protein was separated from excess reactant using the Centricon filtration units, as previously described for alkylating agent 1. Because of the large extinction coefficient of FL492, each retentate was rinsed with fresh HMS using the filtration devices until no FL492 was detected in the filtrate. As previously described, the filtrates were discarded. The retentates were diluted with 390 μL HMS and placed in fresh microfuge tubes. Dilutions of each sample were placed in 96-well UV microplates and the spectra of each sample was recorded using the UV-Vis plate reader (Molecular Devices, Sunnyvale, CA, USA). Bovine serum albumin (BSA, 0.078 nM; Sigma-Aldrich Corp.) was used as a positive control and treated the same way as the lens homogenates. The optical density of each sample at three wavelengths was entered into a spreadsheet. The optical density at 280 nm was used to determine the protein concentration using an extinction coefficient of 40,000 and Beer's law. The concentration of unoxidized protein sulfhydryl groups was determined at 322 nm or 384 nm using the appropriate extinction coefficients for ST322 or CPM384, respectively. The concentration of oxidized protein sulfhydryl groups was determined at 490 to 492 nm using the extinction coefficient for FL492. Protein SS/(Protein SS + Protein SH) × 100%SS was calculated for each lens and recorded in column format in a PRISM file. There were 2 moles of total sulfhydryl groups per mole protein in the older mouse lenses.

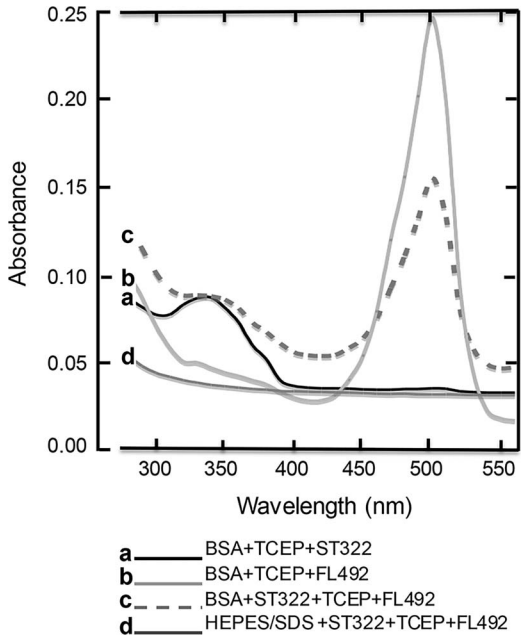


Figure 2. Differential alkylation results for BSA. *Trace a*, all of the cysteine side chains were reacted with ST322. *Trace b*, all of the cysteine side chains were reacted with FL492. *Trace c*, cysteines, not involved in disulfide bonds, were reacted with ST322; then disulfide bonds were reduced with TCEP and alkylated with FL492. *Trace d*, treated blank without BSA.

The method developed for SS and SH quantitation was validated using BSA. Bovine serum albumin has 607 amino acids, 35 of which are cysteines. All 35 cysteine residues reacted with ST322 or FL492 in the presence of TCEP (Fig. 2, Trace a and Trace b, respectively). When alkylated with ST322 before reduction with TCEP and alkylation with FL492, 16 of the 35 cysteines reacted with ST322 (Fig. 2, Trace c). The other 17 were oxidized in the original sample because they reacted with the FL492. Similar results were obtained with CPM384 as alkylating agent 1 (data not included). These results validate the method used to test LA's effects on protein sulfhydryl-group oxidation in mouse lenses.

High-Performance Liquid Chromatography Analysis of Mouse Aqueous Humor Following Topical Ocular Application of LA or LACE

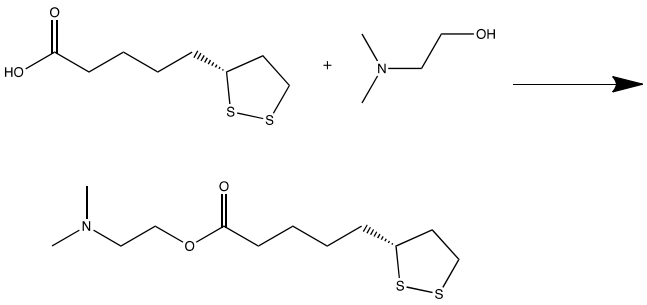
To evaluate ocular penetration of LA or LACE, 5 μL formulated LA or LACE was applied to the right eye (OD) of old mice (four per group) by syringe pump (Harvard Apparatus, Cambridge, MA, USA). Forty-five minutes after topical application, each mouse was euthanized using CO_2 overdose and the aqueous humor was removed from the treated (OD) and untreated (OS) eyes for HPLC analysis.

Samples (10–50 μL) were injected and separated on the Agilent 1050 HPLC system using a C-18 column (250 × 4.6 mm, 5 um; Alltech Associates, Deerfield, IL, USA). The isocratic mobile phase with a flow rate of 1.2 mL per minute consisted of 50% (vol/vol) of solution A (50 mM NaH2PO4, pH 2.7), and 50% (vol/vol) of solution B (60% [vol/vol] acetonitrile and 40% [vol/vol] methanol). The elution buffer was filtered with a 0.45-μm filter before use. The concentrations of the redox active compounds were detected and measured with a Coulochem II multielectrode electrochemical detector (ESA, Inc., Chelmsford, MA, USA). This system consisted of three coulometric electrodes. These were arranged and set at the selected potentials as previously described. The first (1) was a guard cell (Model 5020) that was placed before the autoinjector high-pressure (190–210 bar) fluid inlet and set at 0.90 V. This electrode placed before the column served as a preconditioning electrode to minimize baseline noise. The postcolumn measurement electrode (model 5010A; ESA, Inc.) is made up of two electrodes in series: the first was used to oxidize interference compounds below the redox threshold of the target compounds, and the second electrode measures the sample level. These two electrodes were set as follows: (2) electrode 1 at 0.45 V, and (3) electrode 2 set at 0.85 V. Measurement sensitivity can be adjusted by altering the ESA Coulochem II microamps (μa) full-scale setting in the useful range of 10 to 50 μa found for these studies. Analog data (1 V) were digitized using an HP 39500E AD converter (Agilent, San Jose, CA, USA) and processed using the version 10.1 CHEMSTATION (Agilent) software. The R-isomer of lipoamide (Duke Small Molecule Synthesis Facility, Durham, NC, USA) was used for calibration purposes to relate unknown integrated area to sample amount. The standards provided a linear value of approximately 1500 integrated area-μa/ng (rsquare = 0.996).

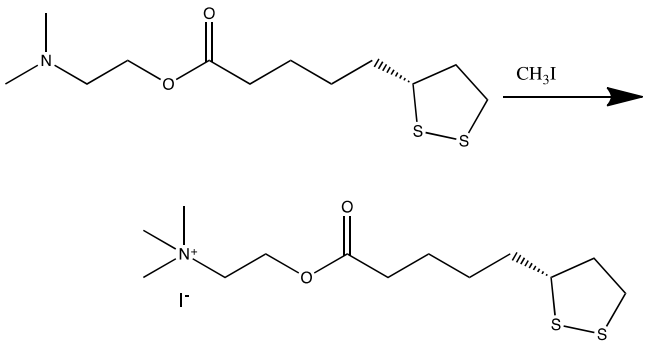
Synthesis of the Iodide Salt of LACE

Lipoic acid is relatively insoluble in aqueous solution. Even in freshly prepared solutions of sodium lipoate, LA precipitates out fairly quickly. To improve on the solubility and lipophilicity for delivery of sufficient LA to the aqueous humor to effect lens elasticity changes, an esterase labile ester of LA with cationic surfactant properties was designed and synthesized in a two-step process as described as Example 3.36 of Patent number US 8,410,162 B2.

In the first step, an ester bond was formed between N,N-dimethylethanolamine and R-LA using dicyclohexylcarbodiimide as the catalyst. The reaction is carried out in dichloromethane containing dimethylpyridine.



The second step is the alkylation of the nitrogen using methyliodide.



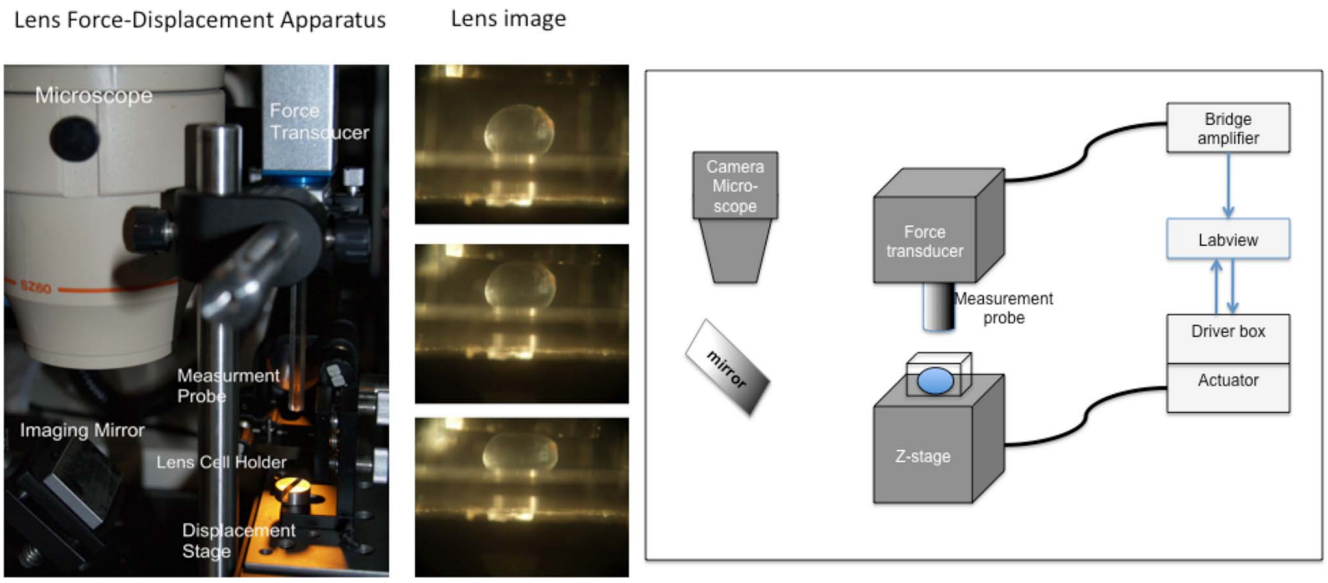


FIGURE 3. Computer-controlled apparatus for lens elasticity measurements and representative photographic results. The Z-stage, lens holder, probe, force transducer imaging mirror, and microscope, depicted in the schematic on the *right*, are labeled in the photograph of the apparatus on the *left*. An example of a lens being studied is presented as three photos. In the *top photograph*, the lens is positioned on Z-stage in modified cuvette with HBSS; the force probe is not in contact with the lens. In the *center photograph*, the force probe is in contact with the top surface of the lens. In the *bottom photograph*, the lens is shown after 405-µm upward stage movement in 15-µm increments. Note that the equatorial diameter has increased and the Z-axis of the lens has decreased.

Mass Spectral Analysis of LA and LACE

Tandem mass spectrometry (MS/MS) studies were performed on a TSQ Quantum Discovery MAX triple stage mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with heated electrospray ionization probe and analyzed with XCALIBUR Software (Thermo Fisher Scientific). Mass spectra ESI-MS (MS) studies were collected in the positive or negative mode, spray voltage 3500, vaporizer temperature 350°, nebulizer 20 units. For CID (collision-induced dissociation) experiments (MS/MS), the argon setting was 1.5 units. For each parent ion, CID experiments were performed at 8 to 11 collision energies between 0 and 50 ev or 0 and −50 ev to identify low-energy, medium-energy, and high-energy fragments.

Computer-Controlled Lens Elasticity Measurements After Topical Ocular Treatment With LACE

Sixteen old mice (age 8 months, Strain C57BL/6J; Jackson Laboratories) were assigned, after acclimation, to one of two groups as follows: group 1, 11 old mice, housed singly, were treated with a sterile formulation containing 5% LACE, right eye (OD). The left eye (OS) was used as an untreated control. For group 1, dosage was a 2.5-µL drop three times per day at 8-hour intervals. Treatment lasted 5 weeks. Formulations were prepared by Ora (Boston, MA, USA). The formulation for the treated eye (OD) contained ethyl pyruvate (0.1%), $NaH_2PO_4 \cdot H_2O$ (0.269%), Na_2HPO_4 (0.433%), hyperomellose (0.2%), NaCl (0.5%), and LACE (5%). Group 2: five treatment-free mice were housed singly for 5 weeks. Group 3: five 8-week old mice were included in the study to define elasticity changes due to age. Mice were euthanized one by one by CO_2 overdose. The lenses were removed within 2 minutes and placed in cuvettes containing HBSS. The lens-containing cuvette was immediately placed on a computer-controlled Z-stage capable of adjustable micron upward movements (Fig. 3). A probe attached to a force monitor was affixed just above the lens to measure and computer-record force at various stage-driven distances. The stage was programmed to first move upward until touching the lens and then reset several microns and slowly continue upward in 15- (young mice) or 25-µm (old mice) increments with a 10-second pause between upward movements.

In cases in which one lens of a pair was damaged during dissection, the elasticity of neither lens of the pair was determined. Four pairs of lenses were excluded from group 1, one pair from group 2, and one pair from group 3.

Data Analysis

For both statistical and regression analyses, PRISM6 for Mac OS 10 (GraphPad Software, Inc.) was used. Column statistics were determined. One-way ANOVA was followed by a Tukey test for comparison of the means. See results section for specific analyses for lens elasticity after treatment in vitro or in vivo.

The LA concentration (X), mean (Y), SD, and n were entered into XY tables for analysis by nonlinear regression. After transforming the [LA] to Log10[LA], the data were fit to a four-parameter Hill dose-response equation,

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left(1 + 10^{((\text{Log}C_{50} - X) \times \text{HillSlope})}\right)},$$

where C_{50} is the concentration for half maximal effect.

RESULTS

Lipoic Acid Increases Lens Elasticity and Reduces Protein Disulfides in a Dose-Dependent Manner In Vitro

The data for the effects of LA on lens elasticity of old mice are presented as a scatter plot (Fig. 4a). One-way ANOVA was

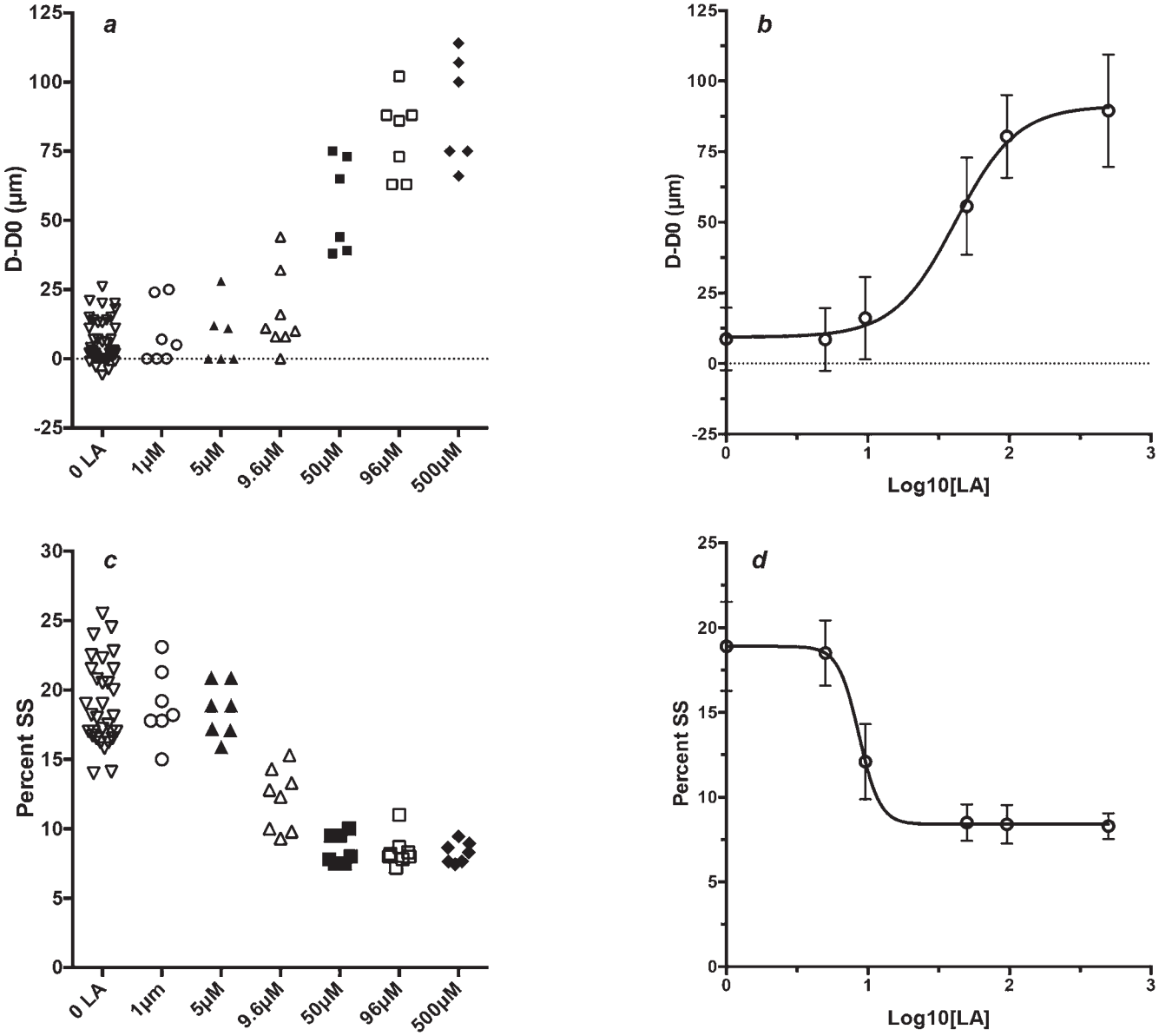


Figure 4. The effect of LA on lens elasticity and lens disulfide levels in vitro. (**a**) Scatter plot of the change in diameter for each individual lens studied after 12-hour exposure, in vitro, to media or media + LA (*x*-axis, µM). (**b**) Dose-response curve of the change in D-D0 (mean ± SD, from data in [**a**]) versus the Log10 of the LA concentration. (**c**) Scatter plot of the percent SS of each individual lens in the study with the LA concentrations (µM) indicated on the *x*-axis. (**d**) Dose-response curve of the change in the percent SS (mean ± SD from data in [**c**]) versus Log10 of the LA concentration (µM). All lenses were from old mice (8 months).

calculated for the data presented. With an effect size of 0.8758, the ANOVA analysis was significant, $F_{6,74} = 86.94$, $P < 0.0001$. To summarize the results of Tukey's multiple comparisons test: (1) means (D-D0) for 0, 1 µM, 5 µM, and 9.6 µM LA-treated lenses are not significantly different from each other but are significantly different from the means (D-D0) for lenses treated with 50 µM, 96 µM, and 500 µM LA; (2) although the 50-µM mean is significantly different from the 96 µM and 500 µM, the 96-µM and 500-µM means are not significantly different from each other. These summarized results of Tukey's test suggest that the effect of LA on mouse lens elasticity is dose dependent.

To test for correlation (i.e., dose-dependence) between LA concentration and elasticity, the mean ± SD was plotted versus the Log10[LA] µM (Fig. 4b). The curve through the experimental points resulted from fitting the data to the four-parameter dose-response Hill equation, where C_{50} is the concentration for half maximal effect.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left(1 + 10^{((\text{Log}C_{50} - X) \times \text{HillSlope})}\right)},$$

Fitted values for *Bottom*, *Top*, $LogC_{50}$, C_{50}, and *HillSlope* are collected in Table 1 along with the 95% confidence intervals (95% CI) for each parameter's value; R^2 for the nonlinear regression analysis was 0.8525. The nonlinear regression analysis results support the dose-dependent nature of LA's effect on mouse lens elasticity.

The effects of LA on the lens protein disulfide levels of old mice are shown in a scatter plot (Fig. 4c). The effect size is 0.8129 for the 1-way ANOVA analysis. The differences among the means were significant $F_{6,68} = 49.25$, $P < 0.0001$. To

Table 1. Fitted Values for the Dose-Response Curves for Elasticity and Disulfides

	Lens Elasticity		% Lens Disulfides, SS/(SH+SS)	
Equation Parameter	**Value**	**95% CI**	**Value**	**95% CI**
Bottom	9.392 μM	0.5139–18.27 μM	8.400%	7.660%–9.140%
Top	91.22 μM	78.27–104.2 μM	18.90%	17.59%–20.21%
LogC_{50}	1.620	1.443–1.797	0.9373	8644–1.010
C_{50}	41.71 μM	27.75–62.71 μM	8.656 μM	7.318–10.24 μM
HillSlope	1.999	0.3995–3.602	−5.881	−12.97–1.211

summarize the results of Tukey's multiple comparisons test of the means: (1) means for %SS 0, 1 μM, and 5 μM are not significantly different from each other but are significantly different from the means for 9.6 μM, 50 μM, 96 μM, and 500 μM LA; (2) there is no significant difference among means for 50 μM, 96 μM, and 500 μM LA, but the 9.6-μM mean is significantly different from the 96-μM and 500-μM means. The results of the Tukey's test indicate that the effect of LA on mouse lens disulfide levels is dose dependent.

The effect of LA on protein disulfide levels is indeed dose dependent in these in vitro lens culture experiments (Fig. 4d). The data, plotted as mean ± SD (%SS) versus LA concentration, were analyzed by nonlinear regression analysis using the four-parameter dose-response Hill equation. Fitted values for Bottom, Top, LogC_{50}, C_{50}, and HillSlope are collected in Table 1 along with 95% CI for each parameter's value. In this instance, the HillSlope is negative, indicating that LA's effect is to decrease the level of disulfides; R^2 for the nonlinear regression is 0.8850.

Note that the C_{50} value for LA's dose-dependent changes in disulfide levels (8.656 μM) is approximately 4-fold less than the C_{50} for LA's effects on lens elasticity (41.71 μM). This may indicate that maximal disulfide reduction is required for elasticity increase, or that specific less-exposed and/or less-reactive disulfides require higher concentrations of LA, or that LA has additional non–disulfide-related mechanisms to increase lens elasticity. These additional mechanisms will be the focus of future studies.

Characterization of the Newly Synthesized LACE

Lipoic acid and its choline ester eluted from the HPLC as a single peak with a retention time of 2.85 ± 0.08 minutes. Like LA, LACE has an absorbance maximum at 330 nm. Serial dilutions of a 0.0292 M solution of LACE were used to determine its molar absorptivity (extinction coefficient) at 330 nm. There was a linear correlation between absorbance at 330 nm (A_{330}) versus LACE concentration ($A_{330} = 120.1[\text{LACE}] + 0.02991$, $R^2 = 0.9981$) with the slope (120.1 ± 2.6) being the molar absorptivity. For comparison, LA has a molar absorptivity at 330 nm of 150.[54,55] The comparison of LA and LACE by MS is shown in Figures 5 and 6 and Table 2.

The parent ion (PI) ($m^2 = 0$, no CID) for LA in the negative ion mode had an m/z of 205.07; the PI for LA in the positive ion mode had an m/z of 205.14. For LA, the PI and four fragments were observed in the negative mode (Table 2, top) and an additional three fragments (Table 2, middle) were identified in the positive ion mode. The three fragments identified in the positive ion mode were reported previously in the Human Metabolome Database.[56–58] The fragments identified in the negative ion mode were reported previously, as well.[59] For LA, the fragmentation starts with the five-membered dithiolane ring (Fig. 5). Having confirmed that the CID-MS-MS protocol led to fragmentation of LA into fragments reported by others, previously, CID-MS-MS was used to further evaluate LACE structure. Four fragments (F1, F2, F3, and F4) were used to identify PI292 as LACE (Fig. 6). The relative abundance of the four fragments was dependent on the collision energy (Table 1, bottom). The structure of the fragments confirmed that PI292 was LACE (Fig. 6). At lower collision energies, the predominant fragment has an atomic mass of 233 Da. This fragment is formed by the loss of the trimethyl amine functional group (atomic mass unit = 59 Da), a result identical to that obtained, previously, for acetylcholine.[60] The dithiolane ring fragments at medium collision energy to F2 and uncharged ethanethiol. Unlike LA, the dithiolane ring is relatively stable even at high collision energies based on the structure of F3.

Topical Ocular Treatment With LACE Increased LA Levels in the Aqueous Humor

Having verified the structure of the synthesized LACE, studies were performed to compare the penetration of LA and LACE. Because 50.0 μM to 96.7 μM medium concentrations were needed to significantly increase the elasticity of mouse lenses in culture, four old mice were treated with 5 μL of a formulation of LA, OD. The formulation consisted of LA (0.121 M, 2.8%), tris(hydroxymethyl)aminomethane for increased LA solubility and pH adjustment, as well as 0.1% benzalkonium chloride (BAC). Benzalkonium chloride is not only a biocide, but also a cationic surfactant believed to increase corneal permeability. Forty-five minutes after topical ocular dosing, the aqueous humor LA concentration reached 17.5 ± 5.6 μM, a level well below that needed to affect elasticity. Forty-five minutes after drop application, after euthanasia, the aqueous humor was collected from the treated eye and analyzed by HPLC.

Topical ocular treatment of four old mice with 5 μL 0.119 M (3.4%) formulation of LACE led to LA levels sufficient to effect disulfide reduction and increase elasticity. Forty-five minutes after topical ocular dosing, LA levels in the aqueous humor were 98.2 μM ± 14.8 μm. These levels were 5.6-fold higher than those when the topical ocular treatment was with the 0.121 M (2.8%) LA formulation. Furthermore, the increased levels of LA in the aqueous humor after topical ocular dosing with LACE indicate that ocular esterases hydrolyze LACE into its component parts: LA and choline.

Topical Ocular Treatment With LACE Increases Lens Elasticity in Mice

Group 1 old mice (age 8 months) were treated topically in the right eye (OD) for 5 weeks with a 5% LACE ophthalmic formulation, as described in the Methods section. Group 2 mice were the same age, but neither eye was treated. Untreated young mice (age 8 weeks) were included as group 3 for comparison to the older mice.

The results for each lens of this study are presented in Figure 7 as force-displacement plots with force (F-F0 [mg]), the dependent variable in these experiments, on the *y*-axis and displacement (Z-Z0 [μm]), the independent variable in these

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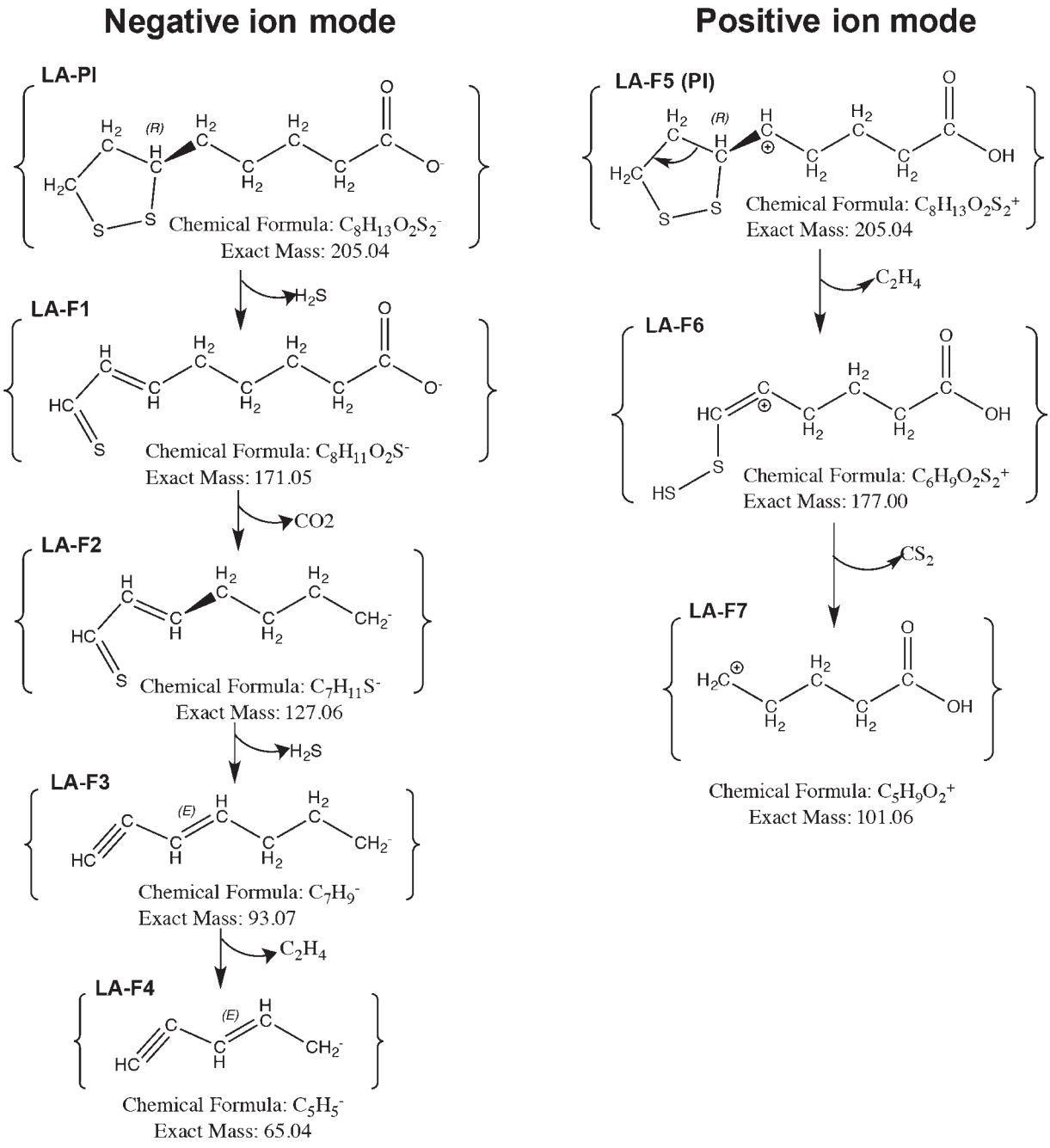


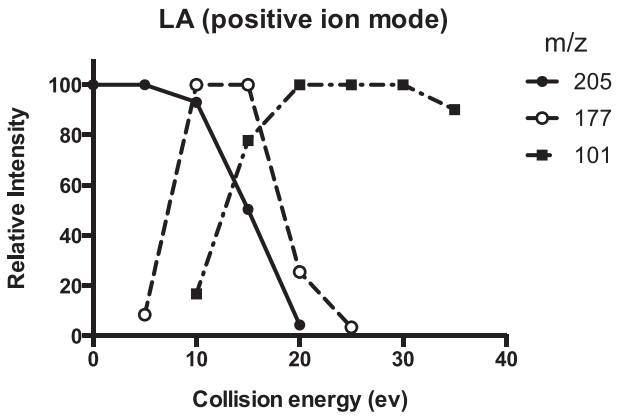
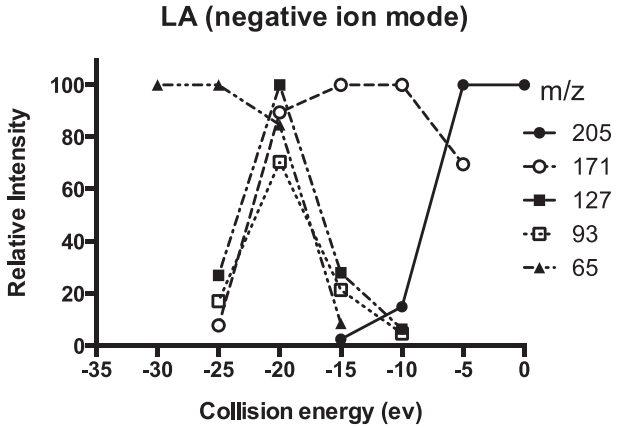
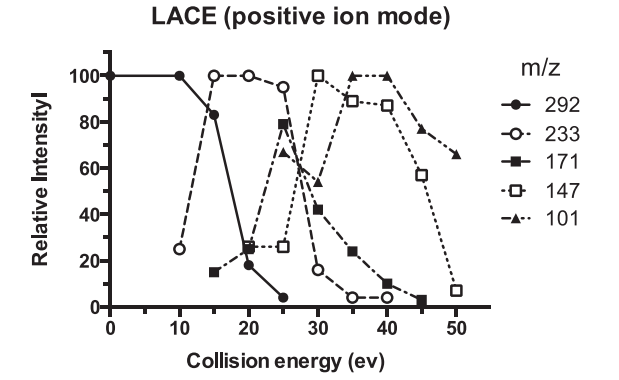
Figure 5. Proposed fragmentation of LA determined by MS. The first step in the fragmentation is the opening of the dithiolane ring by loss of H_2S and C_2H_5 in the negative ion and positive ion modes, respectively.

experiments, on the *x*-axis. With the exception of one lens, treated OD lenses were more elastic than untreated OS lenses in the group 1 mice (Fig. 7, top). For the group 2 and group 3 mice (Fig. 7, middle and bottom, respectively) there was no significant difference between lenses of OD and OS eyes; however, the scatter of the data was greater for the group 2 lenses (9-month-old mice at end of study) than for the group 3 lenses (young mice, age 8 weeks).

For the remainder of the comparisons, means ± SD were determined for group 1 OD lenses ($n = 7$), group 1 OS lenses ($n = 7$), group 2 lenses ($n = 8$), and group 3 lenses ($n = 8$). The resulting force-displacement plots are shown in Figure 8. For the group 1 mice, the lenses of the treated eyes (OD) were more elastic than the lenses of the untreated eyes (OS) (Fig. 8a). This means that, LACE, in vivo, produced a result similar to that of LA in the in vitro experiments (Fig. 4a, 4b). The lenses of the group 2 mice were less elastic than the lenses of the group 3 mice (Fig. 8b). This means that in mice, as in humans, lenses become stiffer with age. The elasticity of lenses of the untreated eyes of group 1 animals and the lenses from untreated group 2 mice were not significantly different (Fig. 8c). This means that there was little to no crossover of LACE into the untreated left (OD) eyes of the group 1 mice. Finally, the lenses of the treated eyes (Fig. 8a, filled circles) were more elastic than those from the untreated group 3 young mice (Fig. 8b, unfilled triangles).

Investigative Ophthalmology & Visual Science

Table 2. The Fragments Identified by MS for LA and for LACE

Fragment Stability Versus Collision Energy	Fragment	AMU* Observed	AMU* Calculated
	PI205 F177 F101	205.14 177.07 101.14	205.04 177.00 101.06
	PI205 F171 F127 F93 F65	205.07 171.09 127.11 93.13 65.05	205.04 171.05 127.06 93.07 65.04
	PI292 F233 F171 F147 F101	292.15 233.07 171.05 147.11 101.05	292.24 233.07 171.05 147.03 101.06

* Atomic Mass Unit (AMU).

Investigative Ophthalmology & Visual Science

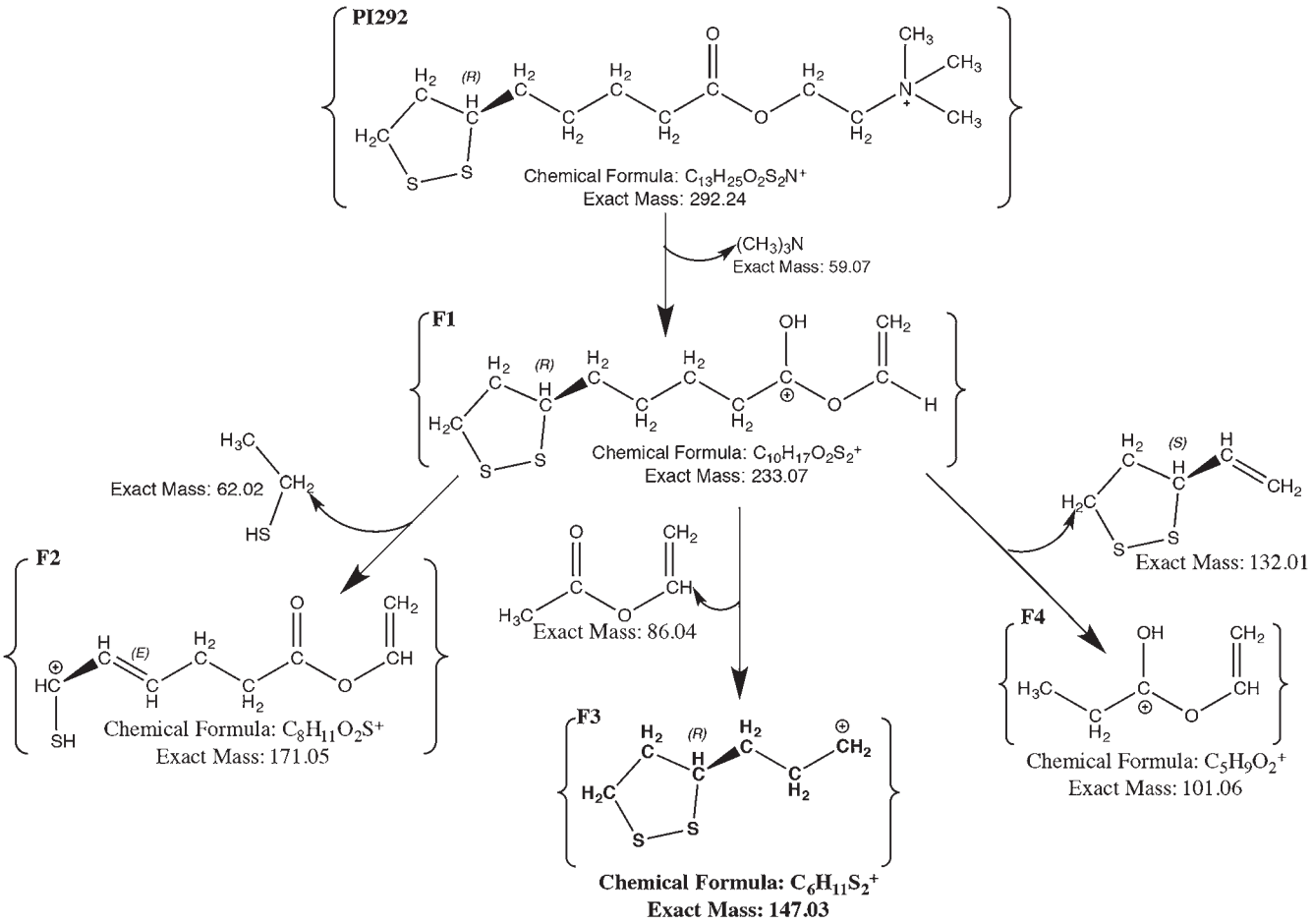


Figure 6. Proposed fragmentation of LACE in the positive ion mode based on the atomic masses of the most prevalent ions. Where F2 forms by break-up of the dithiolane ring, the ring is intact in F3 and totally lost, presumably as a fragment, when F4 is formed.

Discussion

This is the first report to demonstrate that mouse lens elasticity can be increased by treatment with LA. The ex vivo lens culture experiments revealed that the increase in lens elasticity is dependent on the concentration of LA. Along with the concentration-dependent increase in elasticity, LA caused a concentration-dependent decrease in mouse lens protein disulfides. Because the concentration of LA required for half maximal decrease in mouse lens disulfide levels was 4-fold less than the concentration of LA for half maximal increase in mouse lens elasticity, some might conclude that protein disulfides contribute little to mouse lens stiffness.

Despite the differences in the half maximal dose, the reduction of protein disulfides[61] and the elevation of GSH levels[61–63] are the most likely mechanisms by which LA softened the mouse lens. The redox potential of DHLA:LA is –0.32 V.[64] For comparison, dithiothreitol:trans-4,5-dihydroxy-1,2-dithiane, a well-established reducer of protein disulfides, has a redox potential of –0.33 V.[65] Interestingly, GSH is a weaker reducing agent than LA or dithiothreitol. The GSH/GSSG redox potential, in vivo, varies with the cell's status (–0.17 V, –0.20 V, and 0.24 V in apoptotic, differentiating, and dividing cells, respectively).[66] The reduction of PSSG and the clearance pathway for LA would lead to higher GSH levels, the former by disulfide exchange and the latter by increasing intracellular cysteine.

A unique amphipathic cationic surfactant prodrug, LACE was synthesized, characterized, and shown to deliver sufficient levels of LA to the aqueous humor to potentially effect changes in lens elasticity and protein disulfides. Esterase-dependent cleavage of LACE released not only LA but also choline. Choline, an endogenous natural product, is a precursor for the neurotransmitter acetylcholine and the phosphatidylcholine class of lipids. More importantly for the use of LA ocularly, choline catabolism provides a methyl-group donor, N,N-dimethylglycine (betaine), for the conversion of homocysteine to methionine for maintenance of S-adenosylmethionine levels.[67] In other tissues, the first two steps in the clearance of excess LA is reduction to dihydrolipoic acid followed by S-adenosylmethionine–dependent methylation of the SH-groups of dihydrolipoic acid to form 6,8-bismethylthiooctanoic acid.[59,62,68,69]

Most important, topical ocular treatment with LACE significantly increased the elasticity of lenses of 8-month-old mice. The lenses of the treated eyes were more elastic than those from 8-week-old mice. In fact, the lenses of the treated eyes were less stiff than those of the 4-week-old mice described in a previous study.[50] The softening of the mouse lens by LACE treatment occurred through the reduction of intermolecular as well as intramolecular protein disulfides.[29] Earlier studies demonstrated that the mouse lens nucleus has more protein disulfides than free protein sulfhydryl groups (PSH).[48,70] In the mouse lens cortex, PSH predominates. There was little indication of mixed disulfides (PSSG or PSSC) in the mouse lens.[48]

If topical treatment with a LACE formulation were to have a similar effect on human lenses, most likely accommodation would be restored to the presbyope.

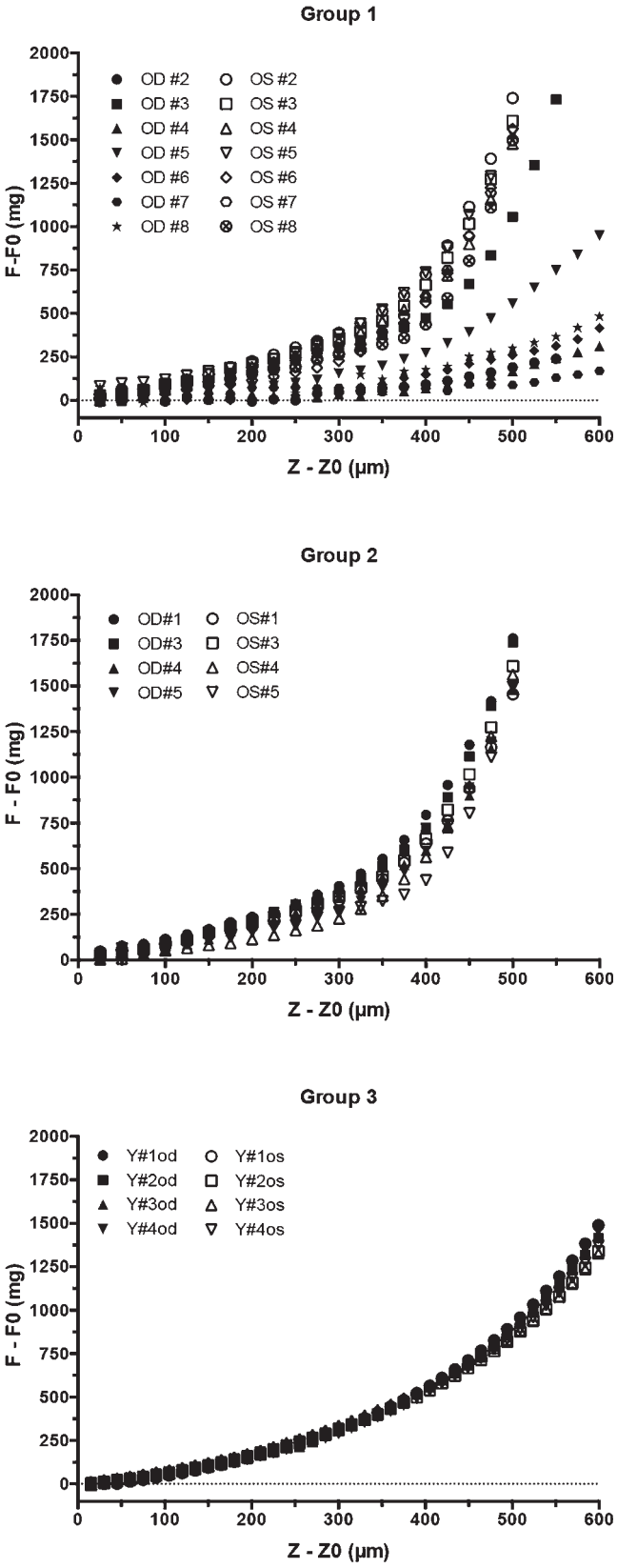


Figure 7. Comparison force-displacement plots of lenses from OD (*filled symbols*) and OS (*unfilled symbols*) eyes. Group 1, old mice (OD treated, OS untreated).

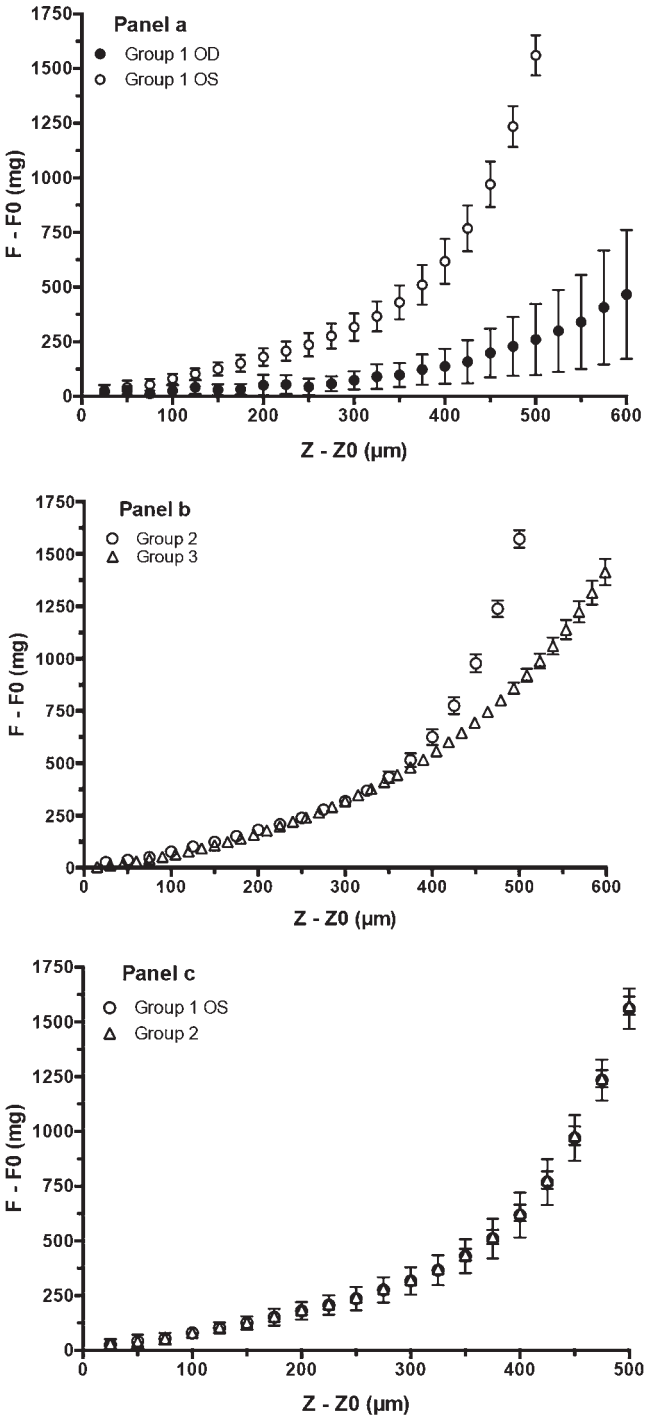


Figure 8. Intergroup and treated versus untreated force-displacement curve comparisons. Force-displacement plots (mean ± SD) for intergroup comparisons (**b**, **c**) and for comparisons of lens elasticity from treated (OD, *filled symbols*) and untreated (OS, *unfilled symbols*) eyes of the group 1 old mice (**a**).

Acknowledgments

Supported in part by Small Business Investigational Research Grant R43 EY022817. Remainder of the funding for these studies was from Encore Vision, Inc.

Disclosure: **W.H. Garner**, Encore Vision, Inc. (C, F, I, R) P; **M.H. Garner**, Encore Vision, Inc. (C, F, I, R) P

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