Inhibition of Crystallin Ascorbylation by Nucleophilic Compounds in the hSVCT2 Mouse Model of Lenticular Aging

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PURPOSE. Senile cataracts are associated with oxidation, fragmentation, cross-linking, insolubilization, and yellow pigmentation of lens crystallins. This process is partially explained by advanced glycation end products (AGEs) from ascorbic acid (ASA), as the authors unequivocally demonstrated in an hSVCT2 transgenic mouse. The authors present the first pharmacologic intervention study against ascorbylation in these mice.

METHODS. Five groups of mice from 2 to 9 months of age (10 mice/group) were fed a diet containing 0.1% (wt/wt) amino-guanidine, pyridoxamine, penicillamine, and nucleophilic compounds NC-I and NC-II. AGEs were determined in crystallin digests using high-performance liquid chromatography, liquid chromatography–mass spectrometry, or gas chromatography–mass spectrometry. Lens protein extract was incubated in vitro with ASA or dehydroascorbic acid.

RESULTS. The ASA level increased approximately 10-fold in all groups and was unaffected by treatment. AGEs were increased several-fold in transgenic compared with control lenses. Body weight, food intake, lenticular glutathione, and glycated lysine level were unaltered. In vitro, all compounds inhibited AGE formation. In vivo, NC-I and NC-II significantly decreased protein fluorescence at λex335/λem385 (P = 0.045, P = 0.017, respectively) and λex370/λem440 (P = 0.029, P = 0.007, respectively). Other inhibitors had no effect. After 7 months, only NC-I and NC-II induced a 50% reduction in pentosidine (P = NS for NC-I; P = 0.035 for NC-II). NC-I also decreased carboxymethyllysine (P = 0.032) and carboxyethyllysine (P = NS). Fluorescent cross-link K2P was decreased by NC-I, NC-II, aminoguanidine, and pyridoxamine (P = NS).

CONCLUSIONS. Pharmacologically blocking protein ascorbylation with absorbable guanidino compounds is feasible and may represent a new strategy for the delay of age-related nuclear sclerosis of the lens. (Invest Ophthalmol Vis Sci. 2008;49:4945–4952) DOI:10.1167/iovs.08-1813

METHODS

Animals

All animal experiments were conducted in accordance with procedures approved by the Case Western Reserve University Animal Care Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed under diurnal lighting conditions and allowed free access to food and water. hSVCT2 transgenic mice were generated as described previously.21 Genomic DNA from mouse tails was isolated and subjected to PCR screening using specifically designed primers. The genetic background of these transgenic mice is C57BL/6 after at least eight generations of cross-breeding with wild-type C57BL/6 mice.
Treatment of hSVCT2 Mice with Candidate Ascorbylation Inhibitors

Pyridoxamine dihydrochloride (P9380), DL-penicillamine (P5125), aminoquinidine (A7259), and the guanidino compounds NC-I and NC-II were purchased from Sigma (St. Louis, MO). The structure is displayed in Figure 1. The mouse diet with 0.1% wt/wt inhibitors was produced by Bio-Serv using standard diet (Isopro 5000 Prolab 5P75; LabDiet, Richmond, IN). Doses of the inhibitors were chosen based on similar studies with nucleophilic AGE inhibitors. Transgenic and age-matched control mice (50/50 male/female), 10 mice per group, were maintained on a standard mouse diet or a special medical diet started at 2 months and continued until 9 months of age. Body weight and food intake were monitored monthly. Nine-month-old mice were killed, and eyes were removed and decapsulated to release the lenses, which were processed for AGE determination.

In Vitro Incubation of Lens Crystallins with Ascorbylation Inhibitors

To best mimic the in vivo formation of ascorbylation products, two models of incubation conditions were tested. Model A consisted of 5 mg calf lens protein homogenate (CLP) incubated with 1 mM dehydroascorbic acid (DHA), with or without 1.5 mM inhibitors, under anaerobic conditions in chelex-treated 5 mM sodium phosphate buffer (pH 7.0) at 37°C for 7 days. DHA and inhibitors were refreshed daily to take into account the short half-life of DHA. Model B consisted of a one-time addition of 25 mM ASA with or without 15 mM inhibitors in aerobic conditions, but with chelex-treated buffer for 7 days to slow down but not eliminate the oxidation of ASA. At the end of the incubations, the proteins were dialyzed against phosphate-buffered saline (PBS) for 24 hours at 4°C, dialyzed twice against water for 48 hours at 4°C, and lyophilized. Half the incubated protein (approximately 2.5 mg) was used for enzymatic proteolysis, as previously described.21 The other half of the protein was used for hydrolysis with 6 N HCl. Enzymatic digest and hydrolysates were used for AGE determination.

Measurement of Ascorbic Acid and Glutathione

Lenses were homogenized in 200 μL of 10% trichloroacetic acid (TCA). The supernatant was used for derivatization with dimethyl-o-phenylenediamine to determine ascorbic acid level and for derivatization with 1-fluoro-2,4-dinitrobenzene to determine glutathione (GSH), level as previously reported.21

Enzymatic Digestion of Lens Proteins

The TCA precipitate of mouse lens protein was washed twice with 500 μL ethyl ether and was allowed to dry at room temperature for 10 minutes. The pellet was reconstituted in an Eppendorf tube with 500 μL of 5 mM argon-exchanged, chelex-treated phosphate buffer (pH 7.0). The protein pellet was enzymatically digested with a series of proteases, as previously described.21 Digestion efficiency varied from 64% to 75%. Corresponding enzyme blanks incubated without added protein were used as background control. After digestion, the samples were reconstituted with water for fluorescence measurement, dried, reconstituted with 0.01 M heptafluorobutyric acid in water, and subjected to HPLC assay for the determination of K2P, as described.21 Protein concentrations of the samples were analyzed by means of a ninhydrin assay expressed as a leucine equivalent after enzymatic digestion and after 6 N HCl hydrolysis to evaluate digestion efficiency. Reported values may underestimate the true AGE content given that at best 80% digestion efficiency was obtained.

Fluorescence Spectroscopy

Fluorescence at λex 370/340 nm and 335/385 nm of the enzymatic lens protein digest was measured with a spectrophurometer (821-F; Jasco, Easton, MD). Data were expressed as fluorescence units per unit protein measured as leucine equivalent.

Determination of Advanced Glycation End Products

K2P, pentosidine, CML, CEL, and furosine were analyzed in this study. The acid-labile K2P was determined in lens protein digest, as reported
in our previous study.\textsuperscript{21} Pentosidine, CML, CEL, and furosine were determined in lens protein acid hydrolysate, as previously reported.\textsuperscript{21}

**Statistical Analysis**

All values were expressed as mean ± SD. The statistical significance of differences in mean values was assessed by repeated-measures ANOVA or Student’s \( t \)-test. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Parameters of Homeostasis**

To better interpret our data, we first determined a number of parameters of homeostasis in the five treatment groups and compared the data with untreated wild-type and control mice. As shown in Fig. 2A, ascorbic acid was elevated in all hSVCT2 mice to levels similar to those of the human lens (2 mM) and were unaffected by treatment. Similarly, lenticular glucose concentrations as reflected by furosine (i.e., fructose-lysine) were not significantly different in any of the groups (Fig. 2B), suggesting that the tested drugs did not impair lenticular glucose levels. Finally, and most important, there was no change in lenticular glutathione concentration (Fig. 2C), which could be a confounding factor for the interpretation of the inhibition data.

**Testing for Ascorbylation Inhibition In Vivo and In Vitro**

The five candidate inhibitors aminoguanidine, pyridoxamine, penicillamine, and the guanidino compounds NC-I and NC-II were given to the mice at a concentration of 0.1% (wt/wt) in powdered laboratory chow. In addition to testing the effects of the inhibitors in vivo, these were also tested in vitro with ascorbic acid (model B) and dehydroascorbic acid (model A). The latter was applied with repetitive supplementation to account for its short half-life. We present the data in Figures 3 and 4 whereby the in vitro data obtained with ASA are displayed in the main figure and those obtained with DHA are shown in the inset.

**Effect of Inhibitors on Protein-Bound Fluorescence at \( \lambda_{	ext{ex}}335/\text{em}385 \, \text{nm} \) and \( 370/440 \, \text{nm} \)**

Although fluorescence was not specific, the similarity in the fluorescence spectra of various synthetic AGEs (including pentosidine and vesperlysine A) with those of human lens proteins supported the assumption that AGEs are the main fluorescent species that increase with age in the human lens.\textsuperscript{22} Usually, fluorescence is monitored for AGEs at \( \lambda_{	ext{ex}}335/\text{em}385 \, \text{nm} \) and \( \lambda_{	ext{ex}}370/\text{em}440 \, \text{nm} \). Both types of protein-bound fluorescence were highly elevated in the lens protein digest from transgenic versus wild-type mice (Figs. 3A, 3C) and in the ASA-incubated calf lens crystallins (Figs. 3B, 3D). After 7 months of intervention, NC-I was able to significantly reduce the fluorescence at both \( \lambda_{	ext{ex}}335/\text{em}385 \, (P = 0.045; \text{Fig. 3A}) \) and \( \lambda_{	ext{ex}}370/\text{em}440 \, (P = 0.029; \text{Fig. 3C}) \). NC-II had a similar effect at both wavelengths (\( \lambda_{	ext{ex}}335/\text{em}385 \, P = 0.017; \lambda_{	ext{ex}}370/\text{em}440 \, P = 0.007; \text{Figs. 3A, 3C}) \). Surprisingly, aminoguanidine, penicillamine, and pyridoxamine showed no fluorescence reduction at either

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/932946/)
In contrast to the in vivo data, in model B (ASA) all inhibitors suppressed both types of fluorescence in vitro, though to varying degrees (Figs. 3B, 3D). Moreover, aminoguanidine and penicillamine, which had no effect in vivo, suppressed fluorescence efficiently in vitro. Interestingly NC-I, NC-II, and pyridoxamine all inhibited fluorescence at both wavelengths to approximately the same extent, but only NC-I and NC-II had a significant effect in vivo. Model A (DHA) behaved similarly to ASA for fluorescence at 385 nm (Fig. 3B, inset), but the latter was curiously not suppressed by the guanidino compounds, including aminoguanidine.

The inhibition pattern of pentosidine formation in vivo (Fig. 3E) is similar to the data in Figure 3C and was significant for NC-II but not for NC-I. This is not entirely surprising because...
the excitation/emission wavelengths are similar for both parameters. Again the most potent inhibitors were NC-I and NC-II, with a positive trend for aminoguanidine. Paradoxically, NC-I and NC-II were relatively weak inhibitors in both in vitro models compared with the other inhibitors (Fig. 3F and inset). These discrepancies raised issues that will be addressed in the Discussion.

The glycoxidation/lipoxidation ascorbylation products CML and CEL were vigorously formed from ASA and DHA (Figs. 4B, 4D, and insets), as previously reported, but only NC-I had significant in vivo suppressive activity toward CML (Fig. 4A). A trend toward the suppression of carboxyethyl-lysine (CEL) was observed (Fig. 4C). An overall similar pattern of CML and CEL inhibition was observed in both models in vitro (Figs. 4B, 4D, 4E).

**Figure 4.** Additional AGE levels in in vitro and in vivo intervention studies. (A) Mouse lens protein CML levels were significantly reduced by NC-I ($P = 0.032$). (B) CLP-bound CML level was inhibited by all five inhibitors during in vitro incubation with ASA or DHA (inset). (C) Mouse lens protein CEL was mildly suppressed by NC-I ($P = 0.032$) compared with regular diet alone. (D) CLP-bound CEL level was inhibited by all five inhibitors during in vitro incubation with ASA. (E) Cross-link K2P content in treated mice was lowered by all inhibitors ($P = 0.03$) except penicillamine. One-way ANOVA was used followed by post-hoc analysis for all comparisons ($n = 10$ per group).
insides), whereby NC-I was the weakest inhibitor. Penicillamine was the most potent in vitro inhibitor with no activity in vivo.

Finally, all inhibitors had a tendency to suppress the fluorophore K2P in vivo (Fig 4E). Statistical significance was not reached because of the high standard errors in the control. No in vitro data could be obtained because of interference with other peaks of ultraviolet (UV) light in the chromatogram. We found no significance differences in animal body weight, food intake, or water intake on treatment with any of the inhibitors (data not shown).

**DISCUSSION**

The availability of an animal model that can rapidly accumulate age-related chemical modifications identical with those occurring in the human lens is crucial to the development of pharmacologic agents that can delay the aging of lens crystallins and perhaps delay cataractogenesis as well. The hSVCT2 mouse unequivocally confirmed that part of the yellowing, fluorescence, and AGEs found in old and cataractous human lens crystallins related to ascorbic acid and its oxidation products.21 This was already postulated many years ago by Bensch,24 Ortwemer,7,12 and us.12 The chemical pathways by which ASA is directly react with nucleophiles25 or it can delactonize into of crystallins with DHA.

Studies revealed that pyridoxamine and aminoguanidine can dramatically inhibit AGE formation in diabetic animals. We also chose penicillamine, a thiol amine and aminoguanidine can block AGE formation, with a guanidino group in the structure that can trap dicarbonyl compounds and can block AGE formation, provisionally named NC-I and NC-II. Guanidino compounds can also serve as free radical scavengers34; increased levels were found in the brains of patients with hyperargininemia.35,36 However, their role in this condition and in healthy humans is still unclear.56

At the outset a major finding in this study was that all five inhibitors showed inhibition of ascorbylation in vitro in both modes, though to varying degrees. Aminoguanidine, pyridoxamine, and penicillamine were the most potent inhibitors for specific AGls (pentosidine, CML, and CEL). The most surprising finding, however, was that NC-I and NC-II, which had the weakest in vitro activity, had the best in vivo activity (notably NC-I).

Of particular interest are the data with K2P. This molecule is a fluorescent and UV-active lysine–lysine cross-link and one of the major modifications in old and cataractous human lenses.37 In our previous study, there was a dramatic increase in K2P between 9 and 12 months of age, suggesting the data might have reached significance if we had extended the study by another 3 months. The uniform trend towards its suppression by all nucleophiles except for penicillamine (Fig. 3C) suggests similar effects would have been observed in vitro. As previously mentioned, however, overlapping UV peaks precluded us from interpreting the in vitro data. Nevertheless, the in vivo suppressive effect of the drug appeared to be strong.

A more difficult issue to address was the discrepancy between the in vivo and in vitro effects of NC-I and NC-II compared with pyridoxamine, aminoguanidine, and penicillamine. The metal chelation ability of the latter three compounds might have contributed to their strong in vitro anti-AGE property, as previously reported by Price et al.38 Another possibility is that NC-I and NC-II have been shown elsewhere to penetrate the blood barrier, likely through an active transport. It may thus be possible that these agents achieved millimolar concentrations, thus providing strong dicarbonyl compound trapping activity. Nevertheless, the fact that aminoguanidine and pyridoxamine lowered K2P suggests that these nucleophiles are also able to reach the lens. It is clear that though we postulate that NC-I and NC-II acted as trapping agents (Fig. 2B), other biologic effects, such as a decrease in reactive oxygen species formation, cannot be excluded.

The overall conclusion from these data is that the existing lenticular concentrations of natural nucleophiles or GSH itself, here determined at approximately 2.5 mM, did not suffice to scavenge the ascorbylation precursors and that the tested experimental drugs most likely acted by trapping the reactive carbonyls originating from ascorbic acid. Although the exact nature of the trapped molecules remains to be identified, this conclusion is supported by the fact that the drugs impaired neither lenticular glucose nor glutathione or ascorbic acid levels. If that had been the case, a very different interpretation would have been needed.

These data may have important translational significance for the potential prevention of senile cataracts. The nuclear fraction of the human lens shows a steady increase in blue and green fluorophores with age that are attributable to UV light-induced tryptophan photodegradation reaction,39 kynurenine oxidation products that bind lens crystallins,40 and nonenzymatic glycation/ascorbylation products.41 Lerman et al.42 proposed that the decreased transmission of visible light in the aging lens and in nuclear cataract is mainly a result of the accumulation of lens fluorogens rather than configurational changes of lens protein. More recently, several glycation experiments have demonstrated that lens crystallin chaperone activity is impaired by certain glycation products.42–45 It is thus possible that the fluorophores act as surrogate markers for the total damage to lens crystallins and thus participate in destabilizing lens crystallins and eventually cataractogenesis. Indeed, Lens Opacities Classification System (LOCS) studies indicate a significant correlation between lens opacity, discoloration, and autofluorescence.44–46 Therefore, the prevention of lens discoloration and fluorogen formation may have the potential to delay lens opacification and cataractogenesis.

The trapping of methylglyoxal by guanidino compounds could have deleterious effects, however, if the reported modification of arginine residues by methylglyoxal improves the chaperone activity of the crystallins.47–48 Further experiments with our animal model are in progress in that regard.

In summary, five potential ascorbylation inhibitors were tested in an hSVCT2 transgenic mouse model of lens chemical aging. Two inhibitors, NC-I and NC-II, were found to significantly block the formation of AGls. NC-I, a guanidino compound capable of being delivered into the lens, may surprisingly reveal itself as a potent anticitaractous agent in clinical studies.

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References


