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Nucleophilic compounds decrease advanced glycation end products (AGEs) from ascorbic acid in the hSVCT2 transgenic mouse model of lenticular aging

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Abstract

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 endproducts (AGEs) from ascorbic acid (ASA), as unequivocally demonstrated in our hSVCT2 transgenic mouse(PNAS 103:16912, 2006). We now present the first pharmacological intervention study against ascorbylation in these mice.

Methods—Five groups of mice (10 mice/group) were fed from two to nine months a diet containing 0.1% (wt/wt) aminoguanidine (AG), pyridoxamine (PM), penicillamine (PA), and nucleophilic compounds NC-I and NC-II. AGEs were determined in crystallin digests using HPLC, LC-MS or GC-MS. I*n vitro* incubations of lens protein extract with ASA or dehydroascorbic aicd (DHA) were also performed.

Results—ASA level increased ~10 fold in all groups and was unaffected by treatment. AGEs were several fold increased in transgenic compared to 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 $\lambda_{ex}335/_{em}385$ (p=0.045, 0.017, respectively) and $\lambda_{ex}370/_{em}440$ (p=0.029, 0.007, respectively). Other inhibitors had no effect. After 7 months, only NC-1 and NC-2 induced a 50 % reduction in pentosidine (n.s, p=0.035 respectively). NC-1 also decreased carboxymethyllysine (CML) (p=0.032) and carboxyethyllysine (CEL) (p= n.s). Fluorescent crosslink K2P was decreased by NC-1, NC-2, AG and PM (p= n.s).

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.

Introduction

Advanced glycation end products (AGEs) are formed non-enzymatically by the reaction of reducing sugars and oxoaldehydes, with amino groups in proteins, lipids and nucleic acids through a series of reactions (Maillard Reaction) and their levels have been documented to increase with age in many tissues[1–3]. Reducing sugars are degraded prior to or during their interaction with proteins in order to form AGEs[4–6], and in fact many AGEs are formed from reactive degradation intermediates such as methylglyoxal and glyoxal. From this point of view, advanced glycation can be considered as a set of posttranslational, nonenzymatic modifications of proteins by reactive carbonyl compounds.

With age, various postsynthetic modifications occur in human lens crystallins. These are particularly associated with the water-insoluble (WI) proteins and are characterized by the presence high levels of yellow chromophores and non-tryptophan fluorescence[7,8],

pigmentation[9], aggregation[10], insolubilization[11], fragmentation, and crosslinking[12]. By and large these modifications are greatly enhanced in senile and brunescent cataractous lenses[13]. Various theories and mechanisms have been hypothesized to explain these changes, including oxygen free radicals and oxidation[14,15], glycation (Maillard reaction)[16] and tryptophan oxidation products[17,18]. In recent years, because of the presence of high concentrations of ascorbate in the human lens, considerable interest has focused on the potential role of ascorbate as a mediator of postsynthetic modifications of crystallins in the aging lens. It was found that many phenomena observed in aging and cataractous lenses could be duplicated by the reaction of lens crystallins with ascorbate[19,20]. This hypothesis has been unequivocally confirmed with our recently established mouse model of lens crystallin aging in which the human Sodium Dependent Vitamin C transporter 2 (hSVCT2) was overexpressed under the control of the mouse αA-crystallin promoter fused to a chick lens δ-crystallin enhancer [21]. With age, there was an increase in ascorbic acid oxidation products and crystallin-bound AGEs pentosidine, carboxymethyllysine (CML), vesperlysine A and K2P. We also noticed that at 12 months mouse lenses had acquired a yellow color similar to that observed in old human lenses. This animal model compressed lens crystallin chemical aging from 70 years in the human to 12 months in the mouse, providing us the opportunity to test potential pharmacological inhibitors of lens crystallin aging by the Maillard reaction *in vivo*.

In the present study, we treated 2 months old transgenic mice with five different candidate inhibitors and analyzed lens crystallin-bound fluorescence and AGEs markers after seven months of treatment. Similar experiments were carried out *in vitro*.

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 use of Animals in Ophthalmic and Vision Research. Animals were housed under diurnal lighting condition 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 8 generations of cross-breeding with wild type C57BL/6 mice.

Treatment of hSVCT2 mice with candidate ascorbylation inhibitors

Pyridoxamine dihydrochloride (P9380), DL-penicillamine (P5125), aminoguanidine (A7259), and the guanidino compounds NC-I and NC-II were all purchased from Sigma Company (St. Louis, MI). The structure is displayed in Figure. 1. The mouse diet with 0.1% w/w inhibitors listed above was produced by Bio-Serv company using standard diet Isopro 3000 (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 special medical diet started at 2 months and continued till 9 months of age. The body weight and food intake were monitored monthly. Nine months old mice were sacrificed, eyes were removed and decapsulated to release the lenses. The lenses were processed for AGE determination as described below.

In vitro incubation of lens crystallins with ascorbylation inhibitors

In order to best mimick the *in vivo* formation of ascorbylation products, two models of incubation conditions were tested. Model A consisted of 5mg of calf lens protein homogenate (CLP) incubated with 1mM of DHA, with or without 1.5mM inhibitors, under anaerobic condition in Chelex treated 5mM 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 one time addition of 25mM ASA with or without 15mM inhibitors in aerobic conditions, but with Chelex treated buffer for 7 days in order to slow down but not eliminate the oxidation of ASA. At the end of the incubations the protein was dialyzed against phosphate buffer saline (PBS) for 24 hours at 4°C, then twice against water for 48 hours at 4°C and lyophilized. Half of incubated protein (~2.5mg) was used for enzymatic proteolysis as previously described [21]. The other half of protein was hydrolysis with 6N HCl. Both enzymatic digest and hydrolysates were used for AGEs determination.

Measurement of Ascorbic acid and Glutathione

Lenses were homogenized in 200μl of 10% trichloroacetic acid (TCA). The supernatant was used for derivatization with dimethyl-*o*-phenylene-diamine 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 500μl of ethyl ether and allowed to dry at room temperature for 10 min. The pellet was reconstituted in an Eppendorf tube with 500μl of 5.0 mM argon-exchanged, Chelex-treated phosphate buffer (pH 7.0). The protein pellet was enzymatic digested with series of protease 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 (HFBA) in water, and subjected to HPLC assay for determination of K2P as described [21]. The protein concentrations of the samples were analyzed by means of a ninhydrin assay expressed as leucine equivalent after enzymatic digestion and also after 6N HCl hydrolysis in order to evaluate the digestion efficiency. The reported values may underestimate the true AGE content since at best 80% digestion efficieny was obtained.

Fluorescence Spectroscopy

The fluorescence at λex/em 370/440nm and 335/385nm of the enzymatic lens protein digest was measured with a spectrofluorometer (821-F; Jasco, Easton, MD). The data were expressed as fluorescence units per unit protein measured as leucine equivalent.

Determination of advanced glycation endproducts

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[21]. Pentosidine, CML, CEL and furosine were determined in lens protein acid hydrolysate as previously reported[21].

Statistical Analysis

All values were expressed as means ±SD. Statistical significance of differences in mean values was assessed by repeated-measures ANOVA or Student's *t* test. *P* values of <0.05 were considered statistically significant.

Results

Parameters of Homeostasis

In order 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 (2mM), 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 importantly, 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 lab 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. Below we present the data in Fig. 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_{ex/em}$335/385 nm and 370/440 nm—Although fluorescence is not specific, the similarity of the fluorescence spectra of various synthetic AGEs (including pentosidine and vesperlysine A) with those of human lens proteins supports the assumption that AGEs are the main fluorescent species which increase with age in the human lens [22]. Usually, fluorescence is monitored for AGEs at $\lambda ex_{/em}$ 335/385nm and $\lambda_{ex/em}$ 370/440nm. Both types of protein-bound fluorescence were highly elevated in the lens protein digest from transgenic vs. wild type mice (Fig. 3A and C), as well as in the ASA incubated calf lens crystallins (Fig. 3B and D). After 7 months of intervention, NC-I was able to significantly reduce the fluorescence at both λex/em 335/385 (p=0.045) (Fig. 3A) and λex/em 370/440 (p=0.029) (Fig. 3C). NC-II had similar effect at both wavelengths (λex/em 335/385, p=0.017; λex/em 370/440, p=0.007) (Fig. 3A and C). Surprisingly, aminoguanidine (AG), penicillamine (PA) and pyridoxamine (PM) showed no fluorescence reduction at neither wavelength (Fig. 3A and C). In contrast to the *in vivo* data, in Model B (ASA) all inhibitors suppressed both types of fluorescence *in vitro*, though to various degrees (Fig 3B and 3D). Moreover, aminoguanidine and penicillamine, which had no effect *in vivo*, suppressed it efficiently *in vitro*. Interestingly NC-I, NC-II and pyridoxamine (PM) all inhibited fluorescence at both wavelengths to the about same extent, but only NC-1 and NC-2 had a significant *in vivo* effect. 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 Fig. 3C. This is not entirely surprising since 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 to the other inhibitors (Fig 3F and inset). These discrepancies raise issues that will be addressed in the Discussion.

As we move to the glycoxidation/lipoxidation ascorbylation products CML and CEL we note that they are vigorously formed from both ASA and DHA (Fig. 4B, 4D and insets) as previously reported[23], but that only NC-I had significant *in vivo* suppressive activity toward carboxymethyllysine (CML) (Fig. 4A)._A trend toward suppression of carboxyethyllysine (CEL) was observed (Fig. 4C). *In vitro*, there was an overall similar pattern of CML and CEL inhibition in both models (insets 4B and 4D), whereby NC-I was the weakest inhibitor. Quite remarkably, PA was the most potent *in vitro* inhibitor with no activity whatsoever *in vivo*.

Finally, all inhibitors had a tendency to suppress the fluorophore K2P *in vivo* (Fig 4E). Statistical significance was not reached due to the high standard errors in the control. No *in vitro* data could not be obtained due to interference with other UV peaks in the chromatogram.

We found no significance differences in animal body weight, food and water intake upon treatment with any of the inhibitors (data no 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 pharmacological agents that can delay the aging of lens crystallins, and perhaps cataractogenesis as well. In that regard the hSCVT2 mouse unequivocally confirmed that part of the yellowing, fluorescence and AGEs found in old and cataractous human lens crystallins is related to ascorbic acid and its oxidation products [21]. This was already postulated many years ago by Bensch, Ortwerth and us[7,12,24]. The chemical pathways by which ASA is thought to generate glycating agents likely initiate from dehydroascorbic acid (DHA), which is highly elevated in the hSVCT2 mouse [21]. DHA can either directly react with nucleophiles[25], or it can delactonize into 2,3-diketogulonate which spontaneously degrades into xylosone and erythrulose[26,27] (Fig. 1B). Our *in vitro* modeling reactions now unequivocally confirmed that most compounds that are elevated in mouse lens can be duplicated by *in vitro* incubation of crystallins with DHA.

To approach the question of pharmacological inhibition, we selected pyridoxamine (PM) and aminoguanidine (AG), ie. two AGE inhibitors that have been extensively studied both *in vitro* and *in vivo*[28–30]. Studies revealed that PM and AG can dramatically inhibit AGE formation in diabetic animals. We also chose penicillamine (PA), a thiol compound that has been shown to inhibit AGE formation in diabetic rat tendons [31] and in cultured rabbit proximal tubular epithelial cells[32], as well as AGE formation in bovine eyes incubated with glucose or glucose-6-phosphate[33]. Finally, we chose two promising nucleophiles with a guanidino group in the structure that can trap dicarbonyl compounds and block AGE formation, provisorily named NC-I and NC-II. Guanidino compounds can also serve as free radical scavengers[34] and increased levels were found in brain from hyperargininemia patients[35,36]. However, their role in this condition as well as in normal humans is still unclear[36].

At the outset a major finding in this study is that all five inhibitors showed inhibition of ascorbylation *in vitro* in both models, though to various degrees. By and large, aminoguanidine, pyridoxamine and penicillamine were the most potent inhibitors for specific AGEs, i.e. pentosidine, CML and CEL. The most surprising finding, however, is 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 crosslink 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 mos of age, suggesting the data might have reached significance if we had extended the study by another three months. The uniform trend toward 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 drugs appears to be strong.

A more difficult issue to address is the discrepancy between the *in vivo* and *in vitro* effects of NC-I and NC-II versus PM, AG and PA. The latter three compounds' metal chelation ability may have contributed toward their strong in vitro anti-AGE property, as previously reported by Price et al. (New Reference: PRICE BAYNES JBC 2001). Another possibility is that NC-

I and NC-II have been shown elsewhere to penetrate the blood barrier, likely via an active transport. It may thus be possible that these agents achieved millimolar concentrations providing thus strong dicarbonyl compound trapping activity. Nevertheless, the fact that AG and PM lowered K2P suggests that these nucleophiles are also able to reach the lens. It is clear that while we postulate that NC-I and NC-II act as trapping agents (Fig. 2B), other biological effects such as decrease in reactive oxygen species formation cannot be excluded at this time.

The overall conclusion from the above data is that the existing lenticular concentrations of natural nucleophiles or GSH itself, here determined at about 2.5 mM, does not suffice to scavenge the ascorbylation precursors, and that the tested experimental drugs most likely act by trapping the reactive carbonyls originating from ascorbic acid. While 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 themselves. If that had been the case, very different interpretation would have been needed.

The above 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[38], kynurenine oxidation products that bind lens crystallins [39] and non-enzymatic glycation/ascorbylation products [40]. Lerman et al. proposed that the decreased transmission of visible light in the aging lens and in nuclear cataract is mainly due to the accumulation of lens fluorogens rather than configurational changes of lens protein[38]. More recently, several glycation experiments have demonstrated that lens crystallin chaperone activity is impaired by certain glycation products[41,42]. 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[43–45]. Therefore, prevention of lens discoloration and fluorogen formation may have the potential to delay lens opacification and cataractogenesis. Intringuingly, however, the trapping of methylgyoxal by guanidino compounds could have deleterious effects, if indeed the reported modification of arginine residues by methylglyoxal improves the chaperone activity of the crystallins[46,47]. Further experiments with our animal model are in progress in that regard.

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

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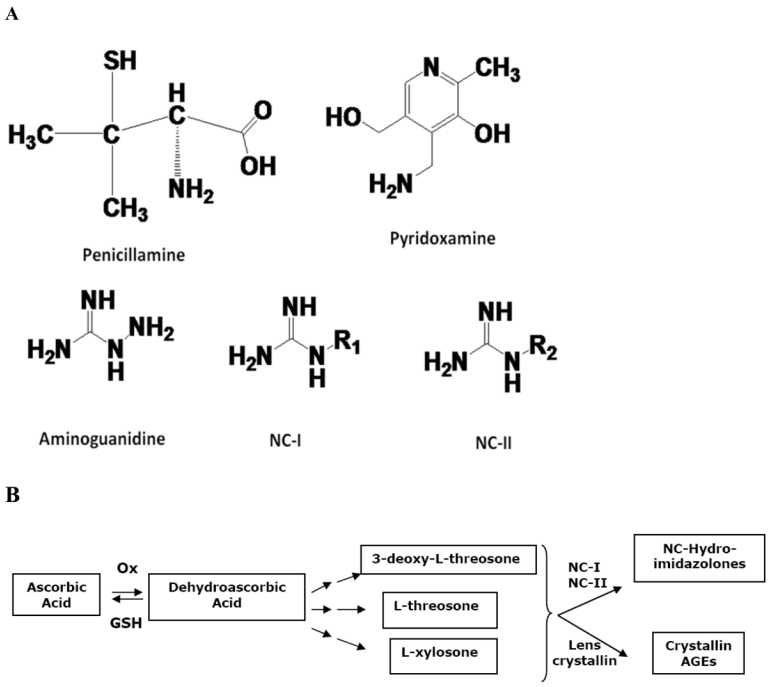

Figure 1.

The structure of tested inhibitors (A) and the potential inhibitory mechanism of action of guanidino compounds against ascorbylation products (B).

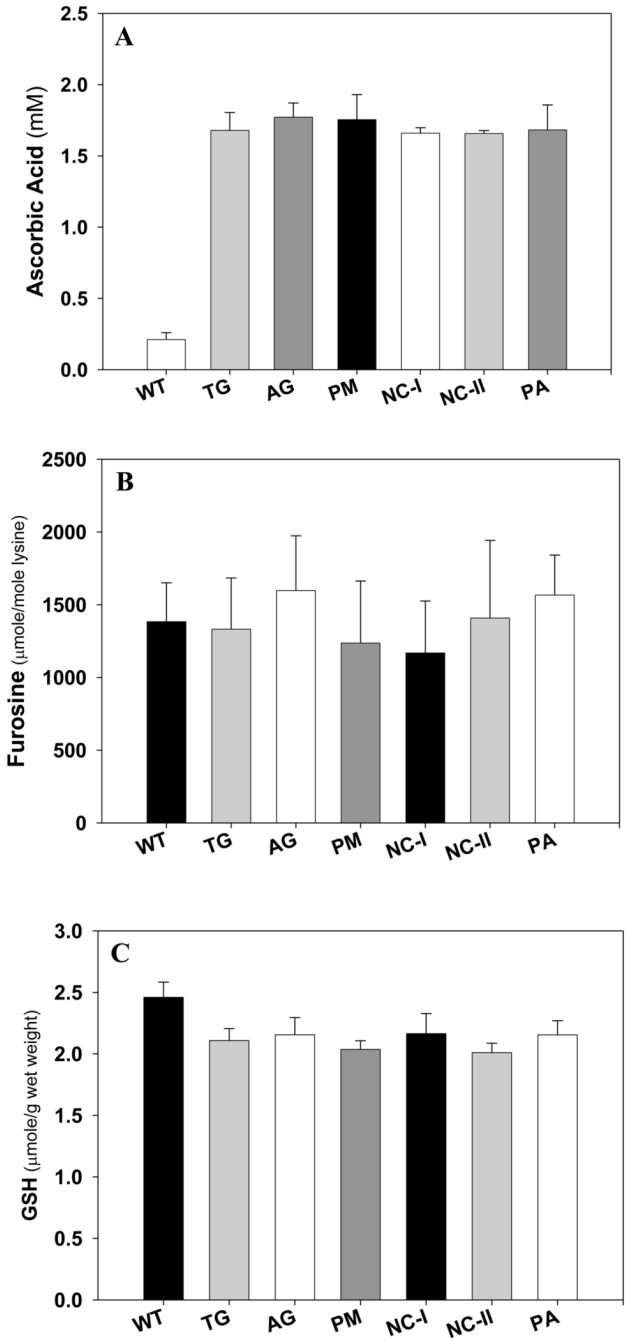

Figure 2.

Lens ascorbic acid, glutathione (GSH) and protein bond furosine level *in vivo*. Mouse lens was homogenized and extracted by 10% TCA. The supernatant was used for GSH and ASA assay and the protein pellet was hydrolyzed for fructosamine (furosine assay), a marker mean glucose levels. (a). Lens ascorbic acid level. (b) lens GSH level. (c) furosine level. Student's *t* test was used for all comparisons. (n=10 mice per group).

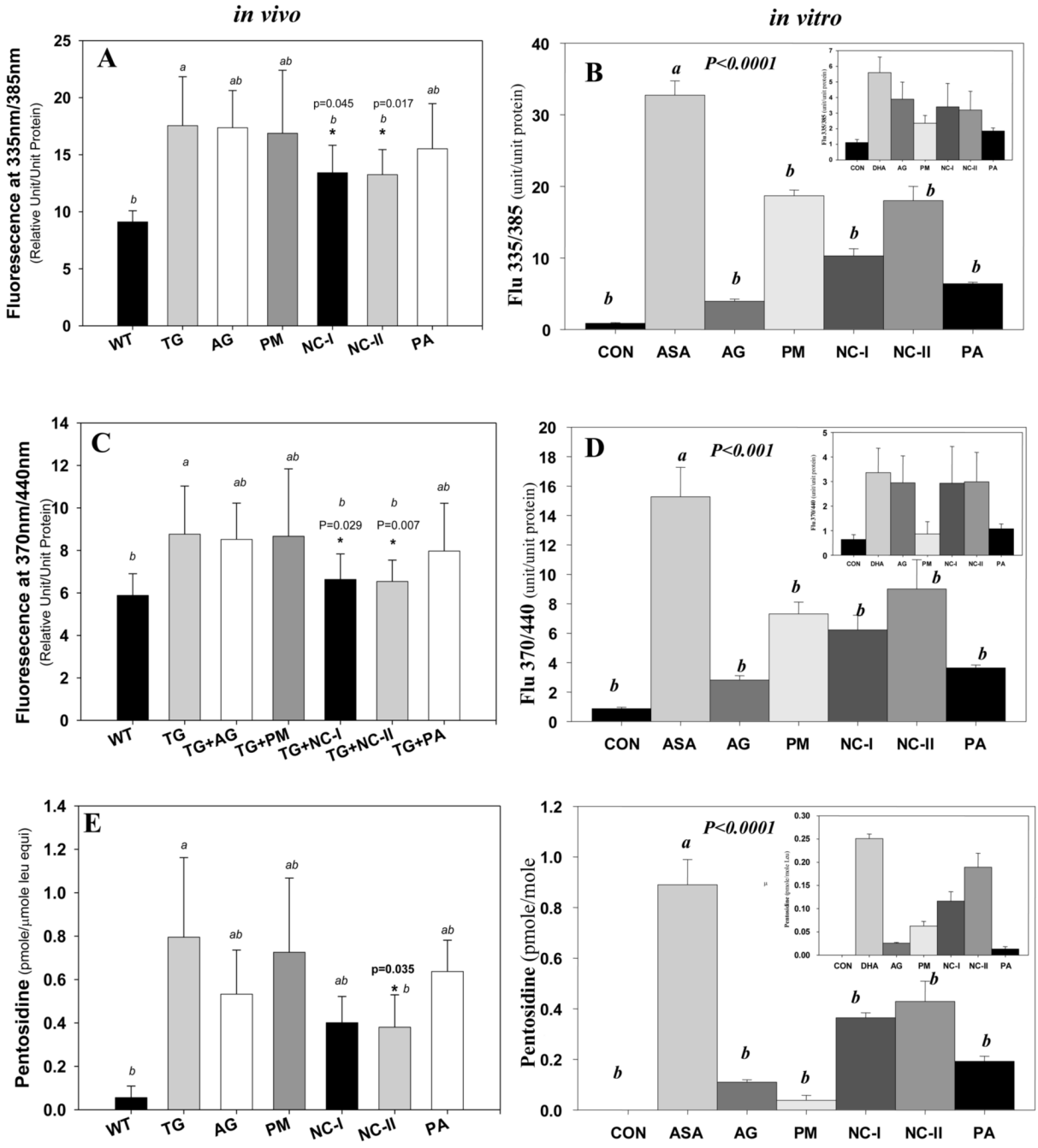

Figure 3.

Levels of protein-bound fluorescence and pentosidine in transgenic mouse lens protein(*in vivo*, panels A,C,E) and calf lens protein extract (CLP) (in vitro, panels B,D,F) incubated with ASA or DHA (inset) with and without inhibitor treatment. (A) and (B) fluorescence at $\lambda_{ex/em}$ 335/385 and at $\lambda_{ex/em}$ 370/440. (E) and (F) pentosidine. One-Way ANOVA was used followed by Post-Hoc analysis for all comparisons (n=10 per group).

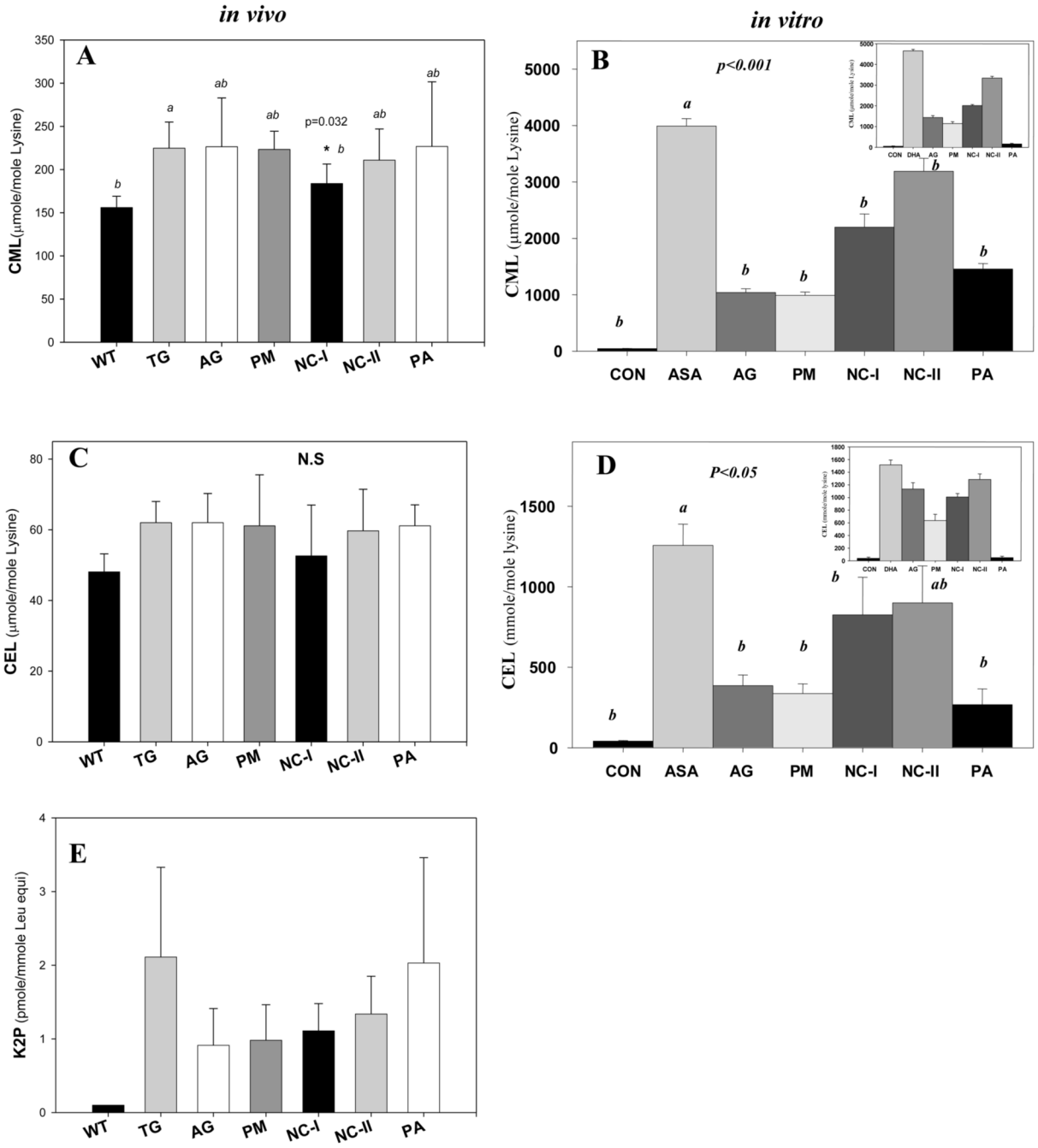

Figure 4.

Additional AGE levels in both *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 = N.S.) vs. 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=N.S) except PA. One-Way ANOVA was used followed by Post-Hoc analysis for all comparisons (n=10 per group).