Ellagic acid, a new antiglycating agent: its inhibition of \(N^\varepsilon-(\text{carboxymethyl})\text{lysine}\)

Puppala MUTHENNA, Chandrasekhar AKILESHWARI and G. Bhanuprakash REDDY

Biochemistry Division, National Institute of Nutrition, Tarnaka, Hyderabad 500 604, India

Non-enzymatic glycation is a complex series of reactions between reducing sugars and amino groups of proteins. Accumulation of AGEs (advanced glycation end-products) due to non-enzymatic glycation has been related to several diseases associated with aging and diabetes. The formation of AGEs is accelerated in hyperglycaemic conditions, which alters the structure and function of long-lived proteins, thereby contributing to long-term diabetic complications. The present study describes AGE inhibition and the mechanism of action of a new antiglycating agent, EA (ellagic acid), a flavonoid present in many dietary sources. Inhibition of AGE formation by EA was demonstrated with different proteins, namely eye lens TSP (total soluble protein), Hb (haemoglobin), lysozyme and BSA, using different glycatifying agents such as fructose, ribose and methylglyoxal by a set of complementary methods. These results suggest that the antiglycatifying action of EA seems to involve, apart from inhibition of a few fluorescent AGEs, predominantly inhibition of CEL \([N^\varepsilon-(\text{carboxyethyl})\text{lysine}]\) through scavenging of the dicarbonyl compounds. Furthermore, MALDI-TOF-MS (matrix-assisted laser-desorption ionisation–time-of-flight MS) analysis confirms inhibition of the formation of CEL on lysozyme on \(\text{in vitro}\) glycation by EA. Prevention of glycation-mediated \(\beta\)-sheet formation in Hb and lysozyme by EA confirm its antiglycatifying ability. Inhibition of glycosylated Hb formation in human blood under \(\text{ex vivo}\) high-glucose conditions signifies the physiological antiglycatifying potential of EA. We have also determined the effectiveness of EA against loss of eye lens transparency through inhibition of AGEs in the lens organ culture system. These findings establish the antiglycatifying potential of EA and its \(\text{in vivo}\) utility in controlling AGE-mediated diabetic pathologies.

Key words: advanced glycation end-product (AGE), ellagic acid, haemoglobin (Hb), lens protein, lens transparency, lysozyme, \(N^\varepsilon-(\text{carboxymethyl})\text{lysine}\).

INTRODUCTION

The global prevalence of diabetes is rising at an alarming rate [1,2]. Chronic hyperglycaemia, with poor glycaemic control, is known to cause various short- and long-term secondary complications, which represent the main cause of morbidity and mortality in diabetic patients [3]. The long-term complications of diabetes are thought to be a result of the accumulation of tissue macromolecules that have been progressively modified by non-enzymatic glycation [4–6]. Glycation is a complex series of covalent chemical reactions between the carbonyl group of reducing sugars and the amino group of proteins. The initial reversible reaction yields a Schiff base, which then undergoes a spontaneous rearrangement to form a more stable Amadori product [7,8]. The latter, over a period of time, undergoes a series of irreversible reactions through highly reactive dicarbonyl intermediates (such as glyoxal, methylglyoxal and 3-deoxyglucose) to form stable heterogeneous adducts called AGEs (advanced glycation end-products) [9,10]. Both the cross-linked and non-cross-linked AGEs are generated through the above processes. \(\text{In vivo}\), a diverse array of AGE products have been detected, some of which are characterized as CML \([N^\varepsilon-(\text{carboxymethyl})\text{lysine}]\), CEL \([N^\varepsilon-(\text{carboxyethyl})\text{lysine}]\), pentosidine, argpyrimidine, imidazolysines, pyrralines, GOLD (glyoxal-derived lysine dimer) and MOLDs (methyl glyoxal lysine dimers) [7,10,11].

AGE formation occurs mainly on long-lived proteins such as the lens crystallins, collagen, myelin, fibronec fibrinogen and other biological molecules such as Hb (haemoglobin), albumin, LDL (low-density lipoprotein)-associated lipids and apoprotein [12–15]. Glycation alters the structure and function of many proteins as it changes the protein charge network and the secondary and tertiary structure, confers resistance to enzymatic digestion, affects enzymatic activity and impairs the receptor recognition [16–19]. In the eye lens, AGEs induce structural destabilization of the protein, which is sufficient to cause the conformational changes that ultimately lead to the formation of HMW (high-molecular-mass) aggregates that scatter the light [16,17]. The formation of AGES plays a key role in the development of several pathophysiologies associated with aging and diabetes such as arthritis, atherosclerosis, chronic renal insufficiency, AD (Alzheimer’s disease), nephropathy, neuropathy and cataract [20–22]. This raises the possibility that inhibition of AGE formation may prevent the progression of diabetic complications. However, designing a drug having anti-AGE activity is a challenge due to the complexity of reactions involved in the formation of AGES. A number of compounds such as aminoguanidine, pyridoxamine, carnosine, ALT-711 (Alagebrium) and phenyl thiouazolium bromide have been investigated in several \(\text{in vitro}\) and \(\text{in vivo}\) studies [23–25]. Aminoguanidine has shown promising results \(\text{in vitro}\), in animal models and in phase III clinical trials. However, the trial was terminated due to safety concerns with major side effects of gastrointestinal disturbance, abnormalities in liver function test, renal neoplasms, production of autoantibodies to histones and anaemia [26–28]. Except pyridoxamine, none of the other

Abbreviations used: AGE, advanced glycation end-product; ALR2, aldose reductase 2; a.m.u., atomic mass unit; BHT, butylated hydroxytoluene; CEL, \(N^\varepsilon-(\text{carboxyethyl})\text{lysine}\); CML, \(N^\varepsilon-(\text{carboxymethyl})\text{lysine}\); DTT, dithiothreitol; EA, ellagic acid; Hb, haemoglobin; HMW, high-molecular-mass; HRP, horseradish peroxidase; KLH, keyhole-limpet (Diodora aspera) haemocyanin; MALDI–TOF–MS, matrix-assisted laser-desorption ionisation–time-of-flight MS; MGO, methyl glyoxal; RBC, red blood cell; TFA, trifluoroacetic acid; TSP, total soluble protein.

1 To whom correspondence should be addressed (email geereddy@yahoo.com).
compounds passed the clinical trials. Hence, the identification and testing of new antiglycating agents with higher levels of efficacy and safety in humans are very much needed.

EA (ellagic acid; 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) is found in numerous fruits and vegetables and other plant foods. EA is known to have antioxidant, anti-inflammatory and anticarcinogenic properties [29–32]. Previously, we have reported that aqueous extracts of some traditional medicines and common dietary agents such as fruits, vegetables, spices and herbs have inhibited or prevented AGE formation under in vitro conditions [33]. We have also shown that the yellow spice turmeric and its active ingredient curcumin modulated the expression of VEGF (vascular endothelial growth factor) in rat retina under hyperglycaemic conditions through inhibition of the formation of AGEs, particularly CML [34]. In addition, some of the above-mentioned dietary agents, such as ginger and cumin, were effective as antiglycating agents under in vivo conditions and thereby delayed diabetic cataract in rats [35,36]. Flavonoids are abundantly found in many of these dietary sources, and EA is one of the commonly found dietary polyphenols. Apart from the greatest sources, such as berries and pomegranate, EA is also present in those dietary sources that we reported to have antiglycating potential such as apples, grapes, orange, guava and cumin [37,38]. Therefore, in the present study, we have investigated the antiglycating activity of EA and its mechanism of action using different in vitro protein glycation systems. Furthermore, the significance and utility of EA antiglycating potential were strengthened in ex vivo physiological conditions.

**EXPERIMENTAL**

**Materials**

Fructose, ribose, glucose, δ-gluconomelactone, lysozyme, Hb, BSA, KLH [keyhole-limpet (Diodora aspera) haemocyanin], RNase, EA, Freund’s complete and incomplete adjuvant, m-amino phenyl boronic acid, HRP (horseradish peroxidase)-conjugated goat anti-rabbit antibody and 3,3′,5,5′-tetramethylbenzidine dihydrochloride and M199 medium modified were obtained from Sigma–Aldrich. Goat anti-mouse antibody conjugated to HRP was from Promega. All other chemicals and solvents used were of analytical grade.

**Preparation of AGE antigens**

AGE antigens were prepared as described previously [17,35,36]. In brief, RNase (25 mg/ml) was incubated with 1 M glucose in 0.2 M sodium phosphate buffer (pH 7.4) containing 0.05% sodium azide at 37 °C for 90 days to prepare AGE-RNase. KLH (50 mg/ml) was incubated with 0.045% glyoxal acid and 0.15 M sodium cyanoborohydride in 0.2 M sodium phosphate buffer (pH 7.8) for 24 h at 37 °C for the preparation of CML-KLH. For MGO (methyl glyoxal)-BSA, BSA (50 mg/ml) was incubated with 0.5 M MGO in 100 mM sodium phosphate buffer (pH 7.5) at 37 °C in the dark for 3 days. Low-molecular-mass reactants and unbound sugars were removed by extensive dialysis.

**Production of polyclonal anti-AGE antibodies**

Antibodies were produced against AGE-RNase, MGO-BSA and CML-KLH by immunizing 3-month-old female New Zealand White rabbits as described previously [33,35,36]. The antiseraum was partially purified by ammonium sulfate fractionation followed by DEAE-Sepharose anion-exchange chromatography to obtain the IgG-rich fraction.

**In vitro glycation of proteins**

Eye lens TSP (total soluble protein) was prepared from 6-month-old goat lenses as described previously [19]. Each 1 ml reaction mixture contained 10 mg of eye lens TSP, 0.2 M phosphate buffer (pH 7.4), 0.2 M fructose, 50 μg of penicillin and streptomycin and 0.01% sodium azide. Similarly, human Hb (20 mg) was glycated with 0.2 M fructose [39]. Reaction tubes were incubated in the dark at 37 °C for 3 weeks under sterile conditions. Each 1 ml reaction mixture for glycation of lysozyme contained 10 mg of lysozyme, 0.2 M phosphate buffer (pH 7.4), 0.4 M ribose, 50 μg of penicillin and streptomycin and 0.01% sodium azide. Reactions tubes were incubated in the dark at 37 °C for 1 week [40]. For glycation of BSA, 50 mg/ml was incubated with 0.8 M glucose under sterile conditions in 1.5 M phosphate buffer (pH 7.4) at 37 °C for 7 days [41].

**AGE inhibition studies with EA**

For inhibition studies, concentrated stocks of EA were prepared in DMSO containing 0.1 M NaOH. Various concentrations of EA (typically 50, 100, 150 and 200 μM) were added to an in vitro protein glycation assay mixture and incubated in the dark at 37 °C for 1–3 weeks as described above.

All of the reaction contents of glycation were filtered through 0.20 μm syringe filters. At the end of the incubation, unbound reactants were removed by extensive dialysis and the protein concentration was determined by the Lowry method [55] using BSA as the standard. The extent of protein glycation in the absence and presence of EA was evaluated by monitoring the subunit profile on SDS/PAGE, AGE-related non-tryptophan fluorescence, protein carbonyl content, affinity chromatography and immunoblotting as described below. The percentage of inhibition with EA was determined, taking the extent of glycation in the absence of the compound as 100%.

**Fluorescence measurements**

Fluorescence measurements were carried out using a Jasco spectrophotometer (FP-6500). For all of the measurements, 0.15 mg of protein/ml in 20 mM sodium phosphate buffer (pH 7.4) was used. AGE-related non-tryptophan fluorescence of glycated protein was monitored by exciting the protein samples at 370 nm, and emission was recorded between 400 and 500 nm. Fluorescence of the glycated BSA samples was measured at the excitation and emission maximum wavelengths of 330 and 410 nm respectively. The spectra or fluorescent readings were corrected with appropriate protein and buffer blanks.

**SDS/PAGE**

The formation of HMW aggregates and cross-linked peptides due to glycation of proteins was monitored by SDS/PAGE (12% gel).

**Glyco-oxidative damage**

Glyco-oxidative damage of proteins was monitored by estimating total protein carbonyls by a method described previously [17,33].

**Immunodetection of AGEs**

The formation of specific AGEs was detected by immunoblotting using anti-CML-KLH, anti-MGO-BSA and anti-AGE-RNase antibodies. Glycated proteins were resolved on SDS/12% PAGE and transferred on to nitrocellulose membrane. The membrane
was incubated for 2 h in blocking buffer containing 5 % (w/v) non-fat dried skimmed milk powder. Subsequently, it was incubated overnight with the respective primary antibodies (CML-KLH, 1:2000 dilution and MGO-BSA/AGE-RNase, 1:1000 dilution) separately. The membrane was then incubated for 2 h with an HRP-conjugated goat anti-rabbit antibody (1:5000 dilution) and detection of the signal was performed using the substrate buffer containing diaminobenzidine and H₂O₂.

**Affinity chromatography**

The extent of glycation of lens proteins in the absence and presence of EA was quantified by phenyl boronate affinity chromatography as described previously [19,35].

**Metal chelation**

Metal-chelating activity of EA was assessed by determining the metal-induced oxidation of ascorbic acid to dihydroascorbic acid using an HPLC method, as described previously [42].

**Post-Amadori inhibition**

BSA (10 mg/ml) was incubated with ribose (0.4 M) at 37°C in 0.4 M phosphate buffer (pH 7.4) for 24 h. Glycation was then interrupted to remove excess ribose and reversible Schiff base by extensive dialysis against 20 mM sodium phosphate buffer at 4°C. The glycated BSA intermediate containing a maximal amount of Amadori product was then incubated at 37°C in the absence or presence of EA for 5 days. This initiated conversion of Amadori intermediates into AGEs and the extent of conversion of Amadori into AGEs [43] were measured by non-tryptophan AGE fluorescence and SDS/PAGE analysis as described above.

**MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) analysis**

Glycation of lysozyme with MGO

Each 1 ml reaction mixture contained 10 mg of lysozyme, 0.2 M phosphate buffer (pH 7.4), 5 mM MGO, 50 μg of penicillin and streptomycin and 0.01 % sodium azide with various concentrations of EA were incubated in the dark at 37°C for 1 week.

**Sample pretreatment and digestion**

Prior to digestion, 50 μl of lysozyme aliquots were precipitated with a 10-fold excess of acetonitrile and the dried precipitates were dissolved in 0.1 ml of 100 mM ammonium bicarbonate buffer (pH 8.5). The free thiol groups were reduced by the addition of 1 μl of 200 mM DTT [dithiothreitol; boiled for 5 min and incubated at room temperature (23°C) for 30 min] followed by the addition of 4 μl of 1 M iodoacetamide to alkylate the free cysteine groups. The solutions were incubated in the dark for 30 min at room temperature and the excess iodoacetamide was quenched by the addition of 4 μl of 1.0 M DTT. Then the sample was dialysed twice for 4 h at room temperature against two 500 ml portions of water, followed by dialysis against 500 ml of 100 mM ammonium bicarbonate buffer (pH 8.2) for an additional 4 h at room temperature. Protein (250 μg) was digested with 5 μg of trypsin at 37°C for 18 h. The digested peptides were purified using C-18 zip tips (Millipore). The peptides eluted in 50 % acetonitrile and 0.1 % TFA (trifluoroacetic acid) were mixed with an equal volume of α-cyano-4-hydroxycinnamate [10 mg/ml in 70 % (v/v) acetonitrile and 0.1 % TFA].

**MS analysis**

An aliquot (1 μl) of the mixture was spotted on to a stainless steel grid, air-dried and subjected to mass determination using MALDI–TOF-MS (ABI 5800). The instrument was equipped with a nitrogen laser and operated in linear mode and in a positive-ion delayed extraction reflector mode. External calibration was performed by using a standard peptide/protein mixture. Usually, each digest was spotted on to at least three individual target positions and 250 individual spectra of each spot were averaged to produce a mass spectrum. Under these conditions, only single-charged states were detected. The monitored mass range was m/z 600–3500.

**CD studies**

CD spectra were recorded at room temperature using a Jasco-810 spectropolarimeter. All spectra are the average of five accumulations. Far- and near-UV CD spectra were recorded using cells of 0.2 and 0.5 cm path length respectively [17,19]. All spectra were corrected for respective blanks. Protein concentrations used for far- and near-UV were 0.15 and 1.5 mg/ml respectively.

**Hb-δ-gluconolactone assay**

The assay involves the determination of glycated Hb (HbA₁c) after incubation with δ-gluconolactone using the ion-exchange chromatography method [44]. This method is specific for the investigation of inhibitors on early glycation product (Amadori) formation. Samples were prepared by mixing 200 μl of whole blood (collected from healthy human volunteers after an overnight fast) with 50 mM δ-gluconolactone in PBS in the absence and presence of different concentrations of EA. Reaction contents were incubated at 37°C for 22 h with occasional brief vortex-mixing. Blood samples were analysed in duplicate for the determination of the percentage of HbA₁c using an ion-exchange system according to the manufacturer’s protocol (Biosystems). The study protocol was approved by the Institutional Ethics Committee of the National Institute of Nutrition and the research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Written consent was obtained from the participants after they were given an explanation of the study details.

**Eye lens organ culture studies**

Three-month-old male Wistar rat lenses were cultured in modified M-199 medium containing 5 mM MGO in the absence and presence of 200 μM EA when present for 3–5 days using a method described previously [17]. After incubation, lenses were homogenized in 20 mM sodium phosphate buffer. The soluble fraction was separated from insoluble protein by centrifugation at 15000 g and the protein concentration was estimated. Immunodetection analysis was carried out using anti-CML and anti-MGO antibodies as described above. Institutional and national guidelines for the use and animal procedures involving animals were followed and all experimental procedures involving animals were approved by the IAEC (Institutional Animal Ethical Committee) of the National Institute of Nutrition. Animals were housed in individual cages in a temperature (22°C)– and humidity-controlled room with a 12-h light/dark cycle. All the animals had free access to water. We adhered to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were sacrificed by CO₂ asphyxiation, and lenses were dissected by the posterior approach [36]. All precautions were taken to ensure
that the animals did not suffer unduly during the experimental procedure.

Statistical analysis

Results are expressed as means ± S.E.M. Data were analysed using SPSS version 15.0 software. Mean values were compared by one-way ANOVA with post-hoc tests of the LSD (least significant difference) method. Heterogeneity of variance was tested by the non-parametric Mann–Whitney test. Differences between comparisons of groups were considered to be significant at $P < 0.05$.

RESULTS

EA showed dose-dependent inhibition of protein glycation and the response varied with the protein and the method used for the detection of glycation. In the case of eye lens TSP, EA decreased AGE fluorescence by approximately 50 and 90% at the concentrations of 50 and 100 μM respectively (Figure 1). When compared with a well-known AGE inhibitor, aminoguanidine, which reduced 60% AGE fluorescence at a concentration of 100 mM [45], the concentration of EA required for exerting a similar antiglycating effect was in the micromolar range. EA inhibited the formation of HMW aggregates upon fructation of TSP (Figure 2A). Glyco-oxidative damage to TSP was also prevented by EA as there was a 50–90% reduction in the formation of protein-carbonyls upon glycation in the presence

![Figure 1](image1.png)

**Figure 1** Effect of EA on AGE fluorescence of eye lens TSP

(A) Representative non-tryptophan AGE fluorescence of eye lens TSP upon *in vitro* glycation in the absence and presence of EA. Trace 1, protein alone; trace 2, protein + 200 mM fructose; trace 3, protein + fructose + 50 μM EA; trace 4, protein + fructose + 100 μM EA. A.u, arbitrary units. (B) AGE-related fluorescent intensity (at 440 nm emission maximum wavelength) in the absence and presence of EA. Bars 1–4 of (B) correspond to traces 1–4 of (A). Data in (B) are means ± S.E.M. for three independent experiments. *Mean values were significantly different from that of bar 2 ($P < 0.05$).

![Figure 2](image2.png)

**Figure 2** Protein cross-linking and protein oxidation of lens TSP

(A) Representative SDS/PAGE profile of eye lens TSP upon *in vitro* glycation in the absence and presence of EA. Lane 1, molecular mass marker; lane 2, protein alone; lane 3, protein + 200 mM fructose; lane 4, protein + fructose + 50 μM EA; lane 5, protein + fructose + 100 μM EA; lane 6, protein + fructose + 150 μM EA. (B) Protein carbonyl content of soluble lens protein on *in vitro* glycation in the absence and presence of EA. Bar 1, protein alone; bar 2, protein + 200 mM fructose; bar 3, protein + fructose + 50 μM EA; bar 4, protein + fructose + 100 μM EA; bar 5, protein + fructose + 150 μM EA. Data in (B) are means ± S.E.M. for three independent experiments. *Mean values were significantly different from that of bar 2 ($P < 0.05$).

of a 100–150 μM concentration (Figure 2B). In order to highlight the antiglycating potential of EA, we have used *in vitro* glycation assays with three other proteins (Hb, lysozyme and BSA). EA suppressed the AGE formation on Hb as monitored by AGE fluorescence of glycated Hb (Figure 3A). In the Hb glycation assay, while fructose reacts with Hb and forms cross-linked tetramers and HMW aggregates above 200 kDa, EA decreased HMW aggregates and recovery of native Hb (Figure 3B). In the lysozyme glycation assay, lysozyme reacts with ribose and forms glycated lysozyme dimers and tetramers. In the presence of an increasing concentration of EA, not only is there a decrease in AGE fluorescence of lysozyme (Figure 4A), but also disappearance of the dimer and tetramer bands of lysozyme as monitored by SDS/PAGE (Figure 4B). Similarly, EA also inhibited the AGE formation on BSA (results not shown). We further investigated the antiglycating activity of EA by quantifying the amount of glycated protein in TSP upon *in vitro* glycation with fructose by phenyl boronate affinity chromatography. In the presence of EA, the percentage of glycated protein in TSP was significantly lower (10%) compared with the amount of glycated protein in the absence of EA (38%; see Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420221add.htm).

AGEs is a collective term referring to a heterogeneous group of chemical structures that range from CML to more complex structures, such as pentosidine or vespertilysine. On the basis of relative ability to reduce glycation on different proteins as assessed by different complementary methods, the antiglycating activity of EA was noteworthy as it prevented AGE formation by more than 50% at a concentration of 100 μM in all methods. However, these methods are surrogate indicators of protein glycation, but not specific to a particular AGE. Hence, we have investigated the
ability of EA to inhibit some of these AGEs by immunodetection. Data obtained with immunoblotting demonstrated the presence of diverse antigenic determinants on the surface of protein. For example, anti-CML-KLH detected cross-linked species of approximately 54 kDa along with HMW aggregates equal to 200 kDa, and some HMW could not enter into the SDS/PAGE stacking gel (Figure 5A). Anti-AGE-RNase demonstrated the presence of cross-linked species between 45 and 54 kDa (Figure 5B). Densitometry analysis indicate that AGE-antigens detected by the anti-CML antibody were more prominent than those detected by anti-AGE-RNase antiserum. Interestingly, EA reduced the formation of the above-mentioned antigenic AGE structures on lens proteins in a dose-dependent manner.

Inhibition of CML or CEL by EA was further confirmed by MALDI–TOF-MS. For this we glycated the lysozyme with MGO. MALDI–TOF-MS of intact lysozyme produced a symmetric singly charged mass peak at 14313 a.m.u. (atomic mass unit; see Supplementary Figure S2 at http://www.BiochemJ.org/bj/442/bj4420221add.htm). However, peak broadening with several unresolved antenna peaks appeared in the mass spectrum of glycated lysozyme. Peak broadening disappeared in the presence of EA and its effects on specific modifications could not be identified in linear mode (Supplementary Figure S2). This necessitated protein digestion prior to the analysis. The MALDI–TOF-MS peptide mass spectrum of lysozyme incubated with MGO in the absence and presence of EA is shown in Figure 6. The peptide masses observed in the mass spectrum were in complete agreement with those of the theoretical peptide masses generated in silico (MS-digest). However, two additional mass peptide peaks (3008 and 3163 a.m.u.) were observed in the glycated lysozyme (Figures 6A and 6B), which are likely to be modifications of peptides of 3008 and 3163 a.m.u. respectively present in the native lysozyme due to the formation of CEL (molecular mass 72 a.m.u.; Figure 6A) and hydroimidazolone (molecular mass 54 a.m.u.; Figure 6B and Table 1). Interestingly, the presence of EA (50 and 200 μM) inhibited the CEL and hydroimidazolone modification as indicated by the decreased abundance of the 3008 and 3216 a.m.u. mass peaks, as indicated by the decrease in area under the curve of mass peaks. MS digest analysis suggests that the CEL modification site lies between amino acids 87 and 114, whereas the hydroimidazolone modification lies between positions 52 and 79 amino acids respectively. The peptide (87–114) is composed of two lysine residues at positions 64 and 78; therefore one of them could have been modified by MGO. Taken together, these results suggest that EA efficiently inhibits the MGO-induced CEL and hydroimidazolone modifications of lysozyme.

We next investigated the antiglycating action of EA as a post-Amadori inhibitor and a metal chelator. However, the presence of EA in the post-Amadori assay did not result in a significant decrease in AGE fluorescence of post-Amadori products (results not shown). Similarly, EA did not inhibit CuCl2-induced oxidation of ascorbic acid (results not shown). These observations further
corroborate the inhibition of CML by EA, but not post-Amadori inhibition. Furthermore, in order to understand the effect of EA on glycation-mediated changes, we have investigated the secondary structure of Hb by CD. Hb shows a maximum negative ellipticity at approximately 208 and 222 nm because of the $\pi-\pi^*$ amide transitions (parallel) and a large maximum positive ellipticity at 193 nm, indicating that Hb is predominantly a $\alpha$-helix protein (Figure 7A; see Supplementary Table S1 at http://www.BiochemJ.org/bj/442/bj4420221add.htm). The far-UV CD signal for fructose-modified Hb showed a decrease in the $\alpha$-helical content, and an increase in the $\beta$-sheet and random coil content. These changes in the secondary structure suggest a partial unfolding of Hb and the formation of random coil structures upon modification. The antiglycating potential of EA became evident from the changes observed in the secondary structure of Hb modified with fructose, as EA at a concentration of 200 $\mu$M reversed the fructose-mediated secondary structural changes in Hb (Figure 7A and Supplementary Table S1). Similar results were observed with lysozyme glycation by ribose (Figure 7B; see Supplementary Table S2 at http://www.BiochemJ.org/bj/442/bj4420221add.htm).

Eventually, the significance of the antiglycating potential of EA was assessed using two different ex vivo models, which mimic the in vivo conditions. In the first model, the ability of EA was determined by estimating the amount of formation of HbA1c in whole blood under ex vivo conditions. HbA1c is an Amadori product formed from the reaction between glucose and the N-terminal valine residue of the $\beta$-chain of Hb. Human RBC (red blood cell) incubated with $\delta$-gluconolactone showed high levels of HbA1c as compared with control RBC (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/442/bj4420221add.htm; Table 2). Interestingly, EA inhibited HbA1c formation in RBC under these conditions. EA might prevent the interaction of the N-terminal amino group of valine of the $\beta$-chain of human Hb with $\delta$-gluconolactone to form an initial Amadori product, thereby preventing the subsequent formation of stable HbA1c through Amadori rearrangement. These results further indicate that EA acts at early stages of glycation. In order to confirm inhibition of AGE formation and to endorse the utility of EA, the eye lens organ culture model was employed. Incubation of rat lens with 5 mM MGO resulted in loss of transparency (opacification) in a time-dependent manner as assessed by the transmission of the light from the grid through the lens (Figure 8). MGO-induced loss of lens transparency is associated with a decrease in soluble protein content and a concomitant increase in the insoluble fraction compared with control lens (results not shown). We have also determined the extent of AGE formation in the lens by immunodetection using anti-CML-KLH and anti-MGO-BSA antibodies. Culturing lens in the presence of MGO led to the accumulation of AGE-modified proteins as shown by immunoblotting with these AGE antibodies (Figure 9). The formation of AGE due to MGO modification of lens proteins may lead to aggregation and insolubilization, finally resulting in cataract formation [19]. However, EA was not only effective in preventing the formation of CML and MGO-AGE in MGO cultured rat lens, but also prevented the loss of lens opacification (Figures 8 and 9).

**DISCUSSION**

Previously we have reported that some dietary sources have the potential to inhibit AGE formation under in vitro conditions and in animal models [33–36]. While characterizing the active principles responsible for the antiglycating action, we realized that, in addition to other flavonoids [45], EA is present in some of these dietary sources and could contribute to antiglycating properties. EA is a phenolic antioxidant and has been reported to have various health benefits [31,46]. In the present study, for the first time, we demonstrated the potential of EA to inhibit or prevent the formation of
Inhibition of advanced glycation endproducts by ellagic acid

Table 1  Identification of the molecular location of MGO modification products in lysozyme

<table>
<thead>
<tr>
<th>Protein</th>
<th>Observed ion</th>
<th>Theoretical peptide mass*</th>
<th>Peptide sequence*</th>
<th>Mass increase</th>
<th>Modification</th>
<th>Glycated residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>3080.76</td>
<td>3008.77</td>
<td>R0/RTPGSRNLCNIPCSALLSSDTASVINCAC114</td>
<td>72</td>
<td>CEL</td>
<td>Lys113</td>
</tr>
<tr>
<td></td>
<td>3216.70</td>
<td>3163.76</td>
<td>K0/KFESNFIQATNHNTDSTGWGLGIDSNRW109</td>
<td>53</td>
<td>Hydroimidazolone</td>
<td>Arg6 or Arg8</td>
</tr>
</tbody>
</table>

*The theoretical peptide masses and corresponding sequences were obtained from the in silico digestion of lysozyme sequence (Pubmed accession no. 11788708) using MS Digest software (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest).

Table 2  Inhibition of the formation of HbA1c in human blood incubated with α-glucuronolactone under ex vivo conditions in the absence and presence of EA

<table>
<thead>
<tr>
<th>Conditions</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control blood</td>
<td>5.73 ± 0.607</td>
</tr>
<tr>
<td>Blood + α-glucuronolactone</td>
<td>8.87 ± 0.506</td>
</tr>
<tr>
<td>Blood + α-glucuronolactone + 100 μM EA</td>
<td>7.23 ± 0.207</td>
</tr>
<tr>
<td>Blood + α-glucuronolactone + 500 μM EA</td>
<td>6.49 ± 0.400*</td>
</tr>
</tbody>
</table>

*Mean values were significantly different from that of blood with α-glucuronolactone in the absence of EA (P < 0.05).

Figure 7  Secondary structure of Hb and lysozyme

(A) Representative far-UV CD spectra of Hb on in vitro glycation in the absence and presence of EA. Trace 1, Hb alone; trace 2, Hb + 200 mM fructose; trace 3, Hb + fructose + 200 μM EA. (B) Representative far-UV CD spectra of lysozyme on in vitro glycation in the absence and presence of EA. Trace 1, lysozyme alone; trace 2, lysozyme + 400 mM ribose; trace 3, lysozyme + ribose + 200 μM EA.

AGEs on different model proteins and its possible mechanism and importance of action. Inhibition of AGE formation by EA was demonstrated with model proteins such as TSP, Hb, lysozyme and BSA using various commonly used glycating agents employing an array of complementary methods. These results suggest that the antiglycating action of EA seems to involve, apart from the inhibition of a few fluorescent AGEs, predominantly inhibition of CML. Prevention of glycation-mediated structural changes in Hb and lysozyme by EA confirm its antiglycating ability. Prevention of formation of HbA1c in blood signifies the physiological function of EA. Furthermore, prevention of loss of eye lens transparency through inhibition of AGEs supports the antiglycating potential of EA in vivo. Since AGEs are the main protagonists of diabetic complications, the antiglycating ability and the potential to ameliorate the diabetic complications make EA a suitable compound for the treatment of AGE-associated pathologies.

Globally, the number of people with diabetes is projected to rise from 171 million in 2000 to 330 million in 2030 [2,47]. Taking these projections into account, diabetes could become a major threat to public health. Although there have been major advances in the control of hyperglycaemia (diabetes) through dietary changes, hypoglycaemic agents, insulin and islet transplantation, the management of long-term complications of diabetes, such as blindness, atherosclerosis, and renal and neurological problems, remain serious problems to be dealt with. Since a considerable amount of evidence has shown the contribution of AGEs to the development of diabetic complications, inhibition of AGEs is considered to be one promising approach for the prevention and treatment of diabetic complications. The currently available knowledge of the mechanisms involved in AGE formation has led to attempts to inhibit the formation of AGEs. A variety of compounds have been investigated and some have shown promising results [24,25,48,49]. Nonetheless, except for one or two, there are not many antiglycating agents available for clinical use. Previous studies have demonstrated the benefits of natural compounds; hence efforts are being made to identify the natural sources of antiglycating agents that can be tested for their therapeutic value against AGE-mediated pathologies [33–36,45].

In the present paper, we describe structurally a new template of antiglycating compound in the form of EA and provide a mechanistic explanation of its action. While EA is known to have various health benefits [29–31], the results described in the present study provide information about a new function of EA. A number of AGEs have been identified by chemical or immunological methods in tissue proteins or in vitro reactions between sugars, peptides and proteins. The data indicated that inhibition of CML formation was more prominent with EA than other AGES. Among all AGEs, CML is most abundant in diabetic patients, and hence EA could be a potential molecule that needs to be explored further for its prospects for diabetic complications. A noteworthy finding is that EA also inhibited the HbA1c formation in human RBCs under conditions that mimic the situation of
Figure 8 Effect of EA on MGO-induced opacification in lens organ culture studies

Rat lenses were cultured in modified M-199 medium as described in the Experimental section. Lens in the medium alone (A), in the presence of 5 mM MGO for 3 days (B), in the presence of 5 mM MGO along with 200 μM EA for 3 days (C), lens in the medium alone for 5 days (D) in the presence of 5 mM MGO for 5 days (E) and in the presence of 5 mM MGO along with 200 μM EA for 5 days (F).

Figure 9 Immunoblot of soluble and insoluble fractions of rat lens with anti-CML (A) and anti-MGO (B) antibodies

Rat lenses were cultured in modified M-199 medium for 5 days as described in the Experimental section. (A) Lane 1, molecular mass markers; lane 2, control lens soluble protein; lane 3, soluble protein of lens incubated with 5 mM MGO; lane 4, soluble protein of lens incubated with 5 mM MGO along with 200 μM EA; lane 5, insoluble protein of lens incubated with 5 mM MGO; lane 6, soluble protein of lens incubated with 5 mM MGO along with 200 μM EA; lane 7, insoluble protein of lens incubated with 5 mM MGO protein along with 200 μM EA.

HbA1c formation in vivo. These findings indicate the potential of EA to prevent and/or inhibit protein glycation. In addition, we have demonstrated the potential of EA against AGE-mediated loss of lens transparency. MGO-induced changes to lens are relevant to the molecular changes that might occur in the diabetic eye lens such as the greater tendency of reactive carbonyls to induce AGE formation, which could increase the scattering of light. These findings provide an indication a possible use of EA for diabetes-mediated pathologies.

Although some flavonoids are shown to inhibit protein glycation [45,50,51], the mechanism seems to be different with different flavonoids. For example, we have recently shown metal chelation, free radical scavenging and post- Amadori inhibition with rutin [45]. However, in the case of EA, antioxidant potential, metal chelation and post-Amadori inhibition do not seem to significantly contribute to the anti-AGE mechanism, but inhibition of CML through a dicarbonyl trap appears to be the main mechanism. In addition, we have also compared the inhibitory potential of glycation with a classical antioxidant BHT (butylated hydroxytoluene) and BHT did not inhibit AGE formation as shown by its inability to suppress the AGE fluorescence and AGE-mediated HMW formation in TSP (results not shown). However, there is a need for developing new antiglycating agents with higher levels of efficacy and without toxic effects for human use. Considering the non-observed-effect level for EA at 5% (3011 mg/kg of body weight per day) in rats [52], its antiglycating activity at low micromolar concentrations is a promising finding. Moreover, EA has been used as food supplement for various health benefits and the antiglycating potential described in the present study merits further attention.

The impact of glycaemic control in the prevention of diabetic complications has been established by studies such as the UK Prospective Diabetes Study and the Diabetes Control and Complications Trial. Nevertheless, perfect glycaemic control is not always possible. Furthermore, the persistence of progression of hyperglycaemia-induced complications during a subsequent period of normal glucose homoeostasis (called memory of glucose toxicity) [53] suggests that exclusive management of glucose can no longer be viewed as sufficient for the control of long-term complications. Hence, agents that can prevent diabetic complications irrespective of glycaemic control would have advantages in the management of secondary complications. In principle, inhibitors of AGE or antiglycating agents can be included in this category. Thus intensive research continues to identify and test both synthetic as well as natural products for their therapeutic value to prevent the onset and/or delay progression of diabetic complications. In addition, accumulation of intracellular sorbitol due to increased ALR2 (aldose reductase 2) activity has been implicated in the development of various secondary complications of diabetes [54]. Therefore inhibition of ALR2 is also one of the approaches for preventing or arresting the progression of diabetic complications. It is interesting to note that EA also inhibited human recombinant ALR2 and suppressed the formation of sorbitol in RBCs under high-glucose conditions ex vivo (G.B. Reddy, unpublished work). Hence, EA might modulate both glycation and polyol pathways because of a cross-talk between these pathways. Thus these multiple properties support EA as a potential candidate for controlling AGE- and ALR2-mediated diabetic pathological conditions in vivo. Together, these results indicate the antiglycating potential of EA and a scope for controlling diabetic pathological conditions in vivo by EA as pharmacological agents or through food sources that are rich in EA.

In conclusion, dietary intervention and the use of functional foods have been shown to have an important role in the management of diabetes and its complications. However, many studies on functional foods are centred on antioxidant, hypoglycaemic and anti-inflammatory effects but not focused on the antiglycating activity. The results of the present study provide a scope for controlling diabetic pathological conditions in vivo by EA as a pharmacological agent or through food sources that are rich in EA. Studies are under way to investigate the potential of EA against diabetic complications in animal models and subsequently in human studies.

AUTHOR CONTRIBUTION

G. Bhanuprakash Reddy conceived and designed the experiments. Puppala Muthenna and Chandrasekhar Akileshwari performed the experiments. Puppala Muthenna, Chandrasekhar Akileshwari and G. Bhanuprakash Reddy analysed the data and wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr Raghu Pulilakhandam and Mr P. Yadagiri Reddy (National Institute of Nutrition, Hyderabad, India) for their help with the MALDI–TOF-MS analysis.


Received 12 May 2011/24 October 2011; accepted 8 November 2011
Published as BJ Immediate Publication 8 November 2011, doi:10.1042/BJ20110846
SUPPLEMENTARY ONLINE DATA

Ellagic acid, a new antiglycating agent: its inhibition of $N^\varepsilon$-(carboxymethyl)lysine

Puppala MUTHENNA, Chandrasekhar AKILESHWARI and G. Bhanuprakash REDDY

Biochemistry Division, National Institute of Nutrition, Tarnaka, Hyderabad 500 604, India

Figure S1  Effect of EA on the amount of glycated protein in eye lens TSP on in vitro glycation as analysed by phenyl boronate affinity chromatography

Table S1  Changes in secondary structural elements of Hb due to non-enzymatic glycation in the absence and presence of EA

Percentages of $\alpha$-helix, $\beta$-sheet and random coil were determined by CD using a k2d2 plot. Data are the means for three independent experiments.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Percentage of $\alpha$-helix</th>
<th>Percentage of $\beta$-sheet</th>
<th>Percentage of random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>75</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>Hb + 200 mM fructose</td>
<td>49</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td>Hb + fructose + 200 $\mu$M EA</td>
<td>60</td>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

Table S2  Changes in secondary structural elements of lysozyme due to non-enzymatic glycation in the absence and presence of EA

Percentages of $\alpha$-helix, $\beta$-sheet and random coil were determined by CD using a k2d2 plot. Data are means for three independent experiments.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Percentage of $\alpha$-helix</th>
<th>Percentage of $\beta$-sheet</th>
<th>Percentage of random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>18</td>
<td>27</td>
<td>55</td>
</tr>
<tr>
<td>Lysozyme + 400 mM ribose</td>
<td>15</td>
<td>31</td>
<td>54</td>
</tr>
<tr>
<td>Lysozyme + ribose + 200 $\mu$M EA</td>
<td>19</td>
<td>26</td>
<td>55</td>
</tr>
</tbody>
</table>

1 To whom correspondence should be addressed (email geereddy@yahoo.com).
Figure S3  Coloration of human blood due to the formation of HbA1c
Tube 1, control blood; tube 2, blood + δ-gluconolactone; tube 3, blood + δ-gluconolactone + 100 μM EA; tube 4, blood + δ-gluconolactone + 500 μM EA.