N^{ε}-(Carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins

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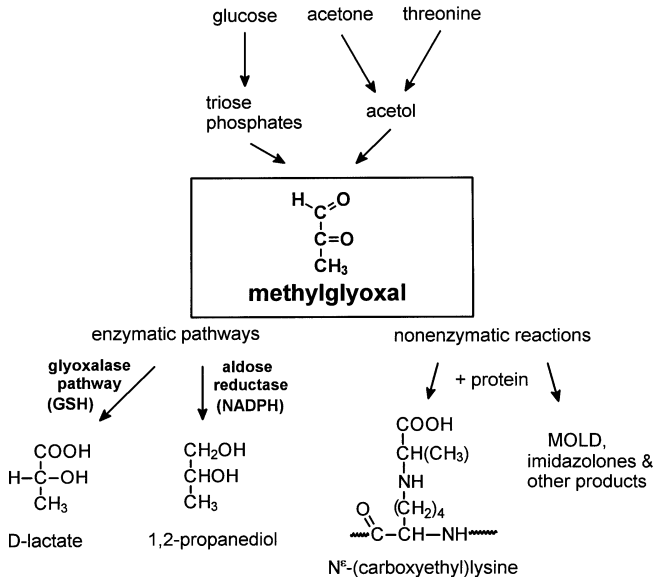
Advanced glycation end-products and glycoxidation products, such as N^{ε}-(carboxymethyl)lysine (CML) and pentosidine, accumulate in long-lived tissue proteins with age and are implicated in the aging of tissue proteins and in the development of pathology in diabetes, atherosclerosis and other diseases. In this paper we describe a new advanced glycation end-product, N^{ε}-(carboxyethyl)lysine (CEL), which is formed during the reaction of methylglyoxal with lysine residues in model compounds and in the proteins RNase and collagen. CEL was also detected in human lens proteins at a concentration similar to that of CML, and increased with age in parallel with the concentration of CML. Although CEL was formed in highest yields during the reaction of methylglyoxal and triose phosphates with lysine and protein, it was also formed in reactions of pentoses, ascorbate and other sugars with lysine and RNase. We propose that levels of CML and CEL and their ratio to one another in tissue proteins and in urine will provide an index of glyoxal and methylglyoxal concentrations in tissues, alterations in glutathione homoeostasis and dicarbonyl metabolism in disease, and sources of advanced glycation end-products in tissue proteins in aging and disease.

INTRODUCTION

The accumulation of Maillard reaction products in tissue proteins is implicated in the development of pathology in aging, diabetes and atherosclerosis [1,2]. Methylglyoxal (MGO) is one of a series of dicarbonyl intermediates, such as glucosone, deoxyglucosones, dehydroascorbate and glyoxal, that have been identified as intermediates in this reaction. MGO is formed non-enzymically by amine-catalysed sugar fragmentation reactions [3,4] and by spontaneous decomposition of triose phosphate intermediates in glycolysis [5]. It is also a product of the metabolism of acetol, an intermediate in the catabolism of both threonine [6] and the ketone body acetone [7] (Scheme 1). MGO reacts rapidly with amino, guanidino and thiol functional groups in proteins, leading to browning, formation of fluorescent products, and cross-linking and denaturation of protein [8,9]. It is detoxified by the glutathione-dependent glyoxalase pathway, which converts MGO into the inert product D-lactate [10,11], and both MGO and acetol are substrates for NADPH-dependent aldose reductase, yielding 1,2-propanediol [12] (Scheme 1). Thornalley and colleagues have reported that the concentrations of MGO and D-lactate and the flux of MGO through the glyoxalase pathway are increased in red cells incubated in hyperglycaemic media *in vitro* [13], and that both MGO and D-lactate concentrations are increased in the blood in diabetes [14,15]. Acetol, the intermediate in the formation of MGO from acetone, and 1,2-propanediol, the product of acetol metabolism by aldose reductase (Scheme 1), are also increased in the plasma of humans during diabetic ketoacidosis [7]. Thus both hyperglycaemia and ketoacidosis may contribute to the increase in MGO concentration in tissues in diabetes, accelerating the browning and cross-linking of proteins and leading to the development of long-term diabetic complications [10–12,16].

In addition to the formation of uncharacterized brown and fluorescent products, MGO is known to react with arginine residues in proteins, yielding an imidazolone compound [9,17]. Related imidazolones are also formed on reaction of proteins with 3-deoxyglucosone, a dicarbonyl sugar formed during



Scheme 1 Sources and reactions of MGO

MGO may be formed by the spontaneous decomposition of triose phosphates or from the metabolism of threonine or acetone. It may be detoxified to D-lactate by the GSH-dependent glyoxalase system, or to 1,2-propanediol via aldose reductase. Otherwise, it may react with amino groups in proteins and other biomolecules to form CEL, methylglyoxal-lysine dimer (MOLD) and unidentified browning products.

Abbreviations used: CEL, N^{ε}-[1-(1-carboxy)ethyl]lysine; 2-CEL, N^{ε}-[1-(2-carboxy)ethyl]lysine; CML, N^{ε}-(carboxymethyl)lysine; MGO, methylglyoxal; MOLD, **m**ethylgly**o**xal-**l**ysine **d**imer (1,3-di-N^{ε}-lysino-4-methylimidazolium salt); SIM-GC/MS, selected ion monitoring gas chromatography/MS; TFAME, *N*-trifluoroacetyl methyl ester; TNBS, trinitrobenzenesulphonic acid.

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Maillard reactions [18,19]. However, neither of these products has been detected in tissue proteins *in vivo*. Brinkmann et al. [20] have also described an imidazolium cross-link structure, **m**ethylgly**o**xal-**l**ysine **d**imer (MOLD), that is formed on reaction of MGO with the peptide mimic hippuryl-lysine. This product was also detected in proteins reacted with MGO *in vitro* [21], and was recently detected in plasma proteins [22].

In the present paper we report on the formation of another lysine derivative, N^{ε}-[1-(1-carboxy)ethyl]lysine (CEL), that is formed during the reaction between MGO and protein. CEL is a homologue of N^{ε}-(carboxymethyl)lysine (CML), an advanced glycation end-product that is formed on reaction of glyoxal [23] or glycolaldehyde [24] with protein and on oxidative cleavage of fructoselysine, the Amadori adduct formed on glycation of protein by glucose [25]. We also show that, like CML, CEL is detectable in human lens proteins, where its concentration increases with chronological age.

A preliminary report of this work has been presented [25a].

MATERIALS AND METHODS

Materials

Unless otherwise stated, reagents were of the highest quality obtainable from Sigma Chemical Co. (St. Louis, MO, U.S.A.), including dihydroxyacetone phosphate lithium salt, D,L-glyceraldehyde 3-phosphate and MGO (40 % aqueous monomer solution). Glucosone was synthesized from the phenylosazone derivative [26] as described by Bayne [27], 3-deoxyglucosone was synthesized as described by Madson and Feather [28], and N^{α}-formyl-lysine was synthesized from lysine formate as described by Hofmann et al. [29]. Bovine pancreatic RNase A (type XII-A) was obtained from Sigma, and rat tail tendon collagen was prepared as described by Fu et al. [30,31]. Normal human lenses were obtained from the South Carolina Lions Eye Bank, Columbia, SC, U.S.A.

CEL was synthesized by reaction of formyl-lysine with pyruvate in the presence of sodium cyanoborohydride ($NaBH_3CN$) (molar proportions 1:1:3) in 0.2 M phosphate buffer, pH 7.4, at 37 °C for 3 days. The solution was adjusted to pH 1.5 with 1 M HCl to discharge residual $NaBH_3CN$, and dried by centrifugal evaporation (Speed-Vac; Savant Instruments, Farmingdale, NY, U.S.A.). Borate was removed as the methyl ester by two rounds of treatment with 0.1 % acetic acid in methanol (65 °C, 5 min), followed by evaporation under a stream of nitrogen (N-Evap; Organomation, Berlin, MA, U.S.A.). The product was deformylated by treatment with 2 M HCl at 95 °C for 2 h, dried by centrifugal evaporation, dissolved in 10 ml of water, adjusted to pH 2.5 and applied to a Dowex-50-H^+ column (10 ml, equilibrated in water adjusted to pH 2.5 with formic acid). The column was washed with 5 column volumes of water, then adjusted to pH 2.5 with formic acid, followed by addition of 5 column volumes of 0.5 M NH_4OH. The NH_4OH eluate, containing CEL and lysine, was evaporated, redissolved in 10 ml of water, adjusted to pH 7.0 and applied to a Dowex-50-NH_4^+ column (10 ml) and eluted with water (5 column volumes) adjusted to pH 6.0 with formic acid. Lysine was retained on the column, and CEL was recovered from the water eluate; the yield was 14 %, based on lysine by amino acid analysis. [$^2H_4$]CEL, the internal standard for selected ion monitoring gas chromatography/MS (SIM-GC/MS) analysis of CEL, was prepared and purified as above, using [$^2H_{4,4,5,5}$]lysine (ISOTEC, Miamisburg, OH, U.S.A.).

Reaction conditions

All reactions were conducted in 0.2 M phosphate buffer, pH 7.4, at 37 °C under air in 12 mm × 75 mm screw-cap test tubes with Teflon-lined caps. Reactions of MGO and other carbohydrates with N^{α}-acetyl-lysine were conducted at equimolar concentrations (100 mM) of sugar and amine for 96 h. Samples were reduced for 4 h at room temperature by addition of a 25-fold molar excess of $NaBH_4$, compared with sugar, in 0.2 M borate buffer, pH 9. Residual $NaBH_4$ was discharged by acidification with 1 M HCl, followed by borate removal and deformylation, as described above. The hydrolysates were dissolved in water and applied to a Dowex-50-H^+ cation-exchange column (1.5 ml, equilibrated with water adjusted to pH 2.5 with formic acid), washed with 5 column volumes of the equilibration buffer, then eluted with 0.5 NH_4OH to obtain lysine and derivatives for amino acid or GC/MS analysis.

Reactions of MGO (15 or 30 mM) with RNase [20 mg/ml, 1.5 mM (= 15 mM lysine); MGO/lysine = 1:1 and 2:1 respectively] were sampled at various times, reduced with $NaBH_4$ as described above, then dialysed against water. Modification of amino groups was measured by the trinitrobenzenesulphonic acid (TNBS) assay [32], and formation of CEL was measured by amino acid analysis.

Rat tail collagen (50–60 mg wet weight) was incubated for 96 h in 2 ml of phosphate buffer containing carbohydrates at 25 mM concentration, with the exception of reactions with glucose, which were conducted for 2 weeks with 250 mM glucose. The supernatant was removed, the collagen washed in deionized water, and the samples worked up by reduction with $NaBH_4$ followed by removal of residual $NaBH_4$ by decantation and washing. The proteins were hydrolysed in 6 M HCl at 110 °C for 24 h for amino acid or SIM-GC/MS analysis.

Amino acid and SIM-GC/MS analysis

Amino acid analysis was conducted by cation-exchange chromatography, using post-column reaction with *o*-phthaldialdehyde [33]. Preparation of *N*-trifluoroacetyl methyl ester (TFAME) derivatives of amino acids and quantitative analysis by GC/MS were conducted as described previously [33]. GC/MS analyses were conducted on a Hewlett–Packard Model 6890 gas chromatograph–mass selective detector system, using a 30 m Rtx-5 capillary column (Restek, Bellefonte, PA, U.S.A.) with the following temperature programme: 2 min at 150 °C, ramp to 180 °C at 6 °C/min, ramp to 300 °C at 15 °C/min, hold for 3 min at 300 °C. SIM-GC/MS was used for quantification based on peak areas of CEL (m/z 379), CML (m/z 392) and lysine (m/z 320), and internal standards [$^2H_4$]CEL (m/z 383), [$^2H_4$]CML (m/z 396) and [$^2H_4$]Lys (m/z 324).

Analysis of human lens and skin collagen samples

Normal human lenses were decapsulated and homogenized in distilled water, as described previously [34]. Samples (100 μl; 3 mg of protein) were reduced for 4 h with 100 μl of 2.5 M $NaBH_4$ in 0.1 M NaOH and processed for GC/MS analysis, as described above for RNase reaction mixtures. Human skin collagen was prepared by scraping to remove adventitious tissue, followed by extraction at 4 °C for 24 h each with 1 M NaCl in PBS, 0.5 M acetic acid and chloroform/methanol (2:1, v/v), as described previously [33].

RESULTS

Formation of CEL in reactions of MGO with N^{α}-acetyl-lysine

We studied the reaction of MGO with acetyl-lysine in order to identify possible products of the reaction between MGO and lysine residues in proteins. Incubations were conducted at equimolar concentrations of MGO and acetyl-lysine under aerobic conditions. Following acid hydrolysis (6 M HCl; 24 h at 110 °C), two acid-stable products were detected by amino acid analysis, one eluting as a neutral amino acid at 33 min (between valine and methionine) and the other eluting as a basic amino acid at 52 min (between lysine and arginine) (Figure 1A). The product at 52 min has been identified as the di-lysine imidazolium cross-link MOLD [20,21]. The compound eluting at 33 min was identified as CEL, based on its co-elution with authentic CEL prepared by chemical synthesis and by comparison of the mass spectrum of its TFAME derivative with that of authentic CEL (Figure 1B). The D- and L-stereoisomers of CEL were not resolved by either amino acid or GC/MS analysis. The yield of CEL was approx. 1.8 %, based on the initial lysine concentration. Approx. 25 % of total lysine was modified during the reaction, so that CEL accounted for about 7.5 % of lysine modification.

Formation of CEL in reactions of MGO with RNase

To determine whether CEL is also formed during the reaction of MGO with proteins, we studied the reaction of MGO with the model protein RNase under physiological conditions. RNase, which has 10 lysine residues including the N-terminal amino acid, was treated with MGO such that the molar ratio of MGO/Lys was 1:1 or 2:1. As shown in Figure 2, CEL was readily detected in MGO-modified RNase by both amino acid analysis (Figure 2A) and SIM-GC/MS (Figure 2B). The products eluting between Phe and Lys, identified by asterisks in Figure 2(A), are the imidazolone adducts formed between MGO and arginine (4 mol of Arg/mol of RNase); several peaks are observed because these adducts equilibrate among four epimeric keto-enol isomers [17–19,35]. The modification of RNase by MGO at a 1:1 molar ratio of MGO/RNase (results not shown) yielded similar amounts of CEL and imidazolium adducts, accompanied by the modification (loss) of 4 mol of Lys and 4 mol of Arg per mol of RNase by amino acid analysis. These results suggest that MGO reacts more rapidly with Arg than with Lys, although the yields of CEL and imidazolium compounds accounted for less than 10 % of the loss of Lys or Arg.

The rate of modification of lysine residues in RNase was measured by the TNBS reaction (Figure 3, upper panel), and the rate of formation of CEL in RNase was measured by SIM-GC/MS (Figure 3, lower panel). Both the modification of lysine residues and the formation of CEL in RNase proceeded rapidly and were dependent on time and MGO concentration, but tended to plateau after 1 day. The half-life of MGO in the reaction mixture was 3–4 h (results not shown), suggesting that some CEL was formed gradually from protein-bound precursors. Treatment with a 1:1 molar ratio of MGO/lysine led to modi-

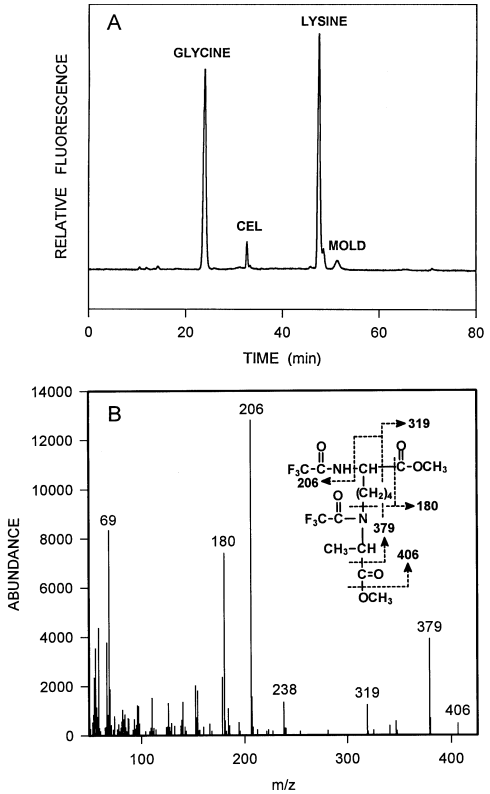

Figure 1 Formation of CEL during the reaction of MGO with N^{α}-acetyl-lysine

(**A**) MGO and N^{α}-acetyl-lysine were reacted at equimolar (25 mM) concentrations in 0.2 M phosphate buffer, pH 7.4, for 7 days at 37 °C. The reaction mixture was processed for amino acid analysis as described in the Materials and methods section. In addition to CEL and lysine, the elution times of glycine (from the internal standard *N*-acetylglycine) and MOLD are indicated on the chromatogram. (**B**) Mass spectrum of the TFAME derivative of CEL, obtained by electron-impact-ionization GC/MS.

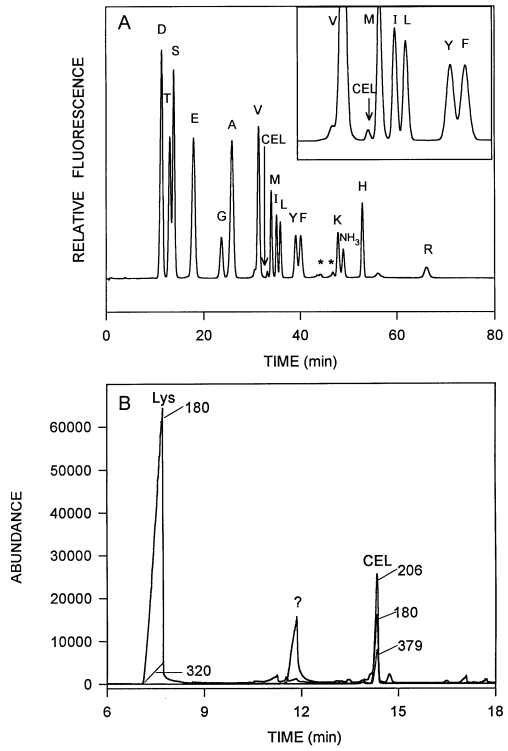

Figure 2 Modification of RNase by MGO

(**A**) Amino acid analysis of RNase (1.5 mM) reacted with MGO (30 mM) for 96 h (see Figure 3). The inset shows an expansion of the region between 28 and 42 min. Amino acids are denoted using the one-letter code; the asterisks indicate the imidazolone adducts formed between MGO and arginine. (**B**) SIM-GC/MS analysis of the sample in (**A**), showing ions recorded for lysine (*m/e* 180 and 320) and CEL (*m/e* 180, 206 and 379).

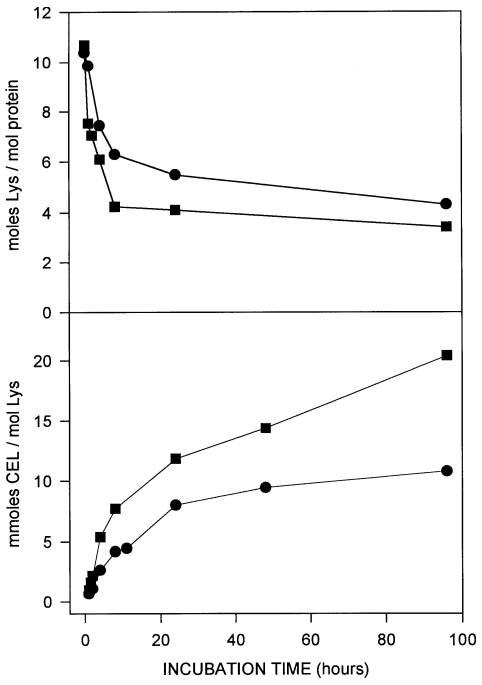

Figure 3 Kinetics of the modification of amino groups (upper panel) and the formation of CEL (lower panel) during the reaction of MGO with RNase

RNase (1.5 mM; = 15 mM lysine) was reacted with 15 mM (●) or 30 mM (■) MGO, as described in the Materials and methods section. Modification of amino groups was measured by the TNBS assay [32], and CEL was measured by SIM-GC/MS.

fication of approx. four of the 10 lysine residues in RNase, but yielded only about 10 mmol of CEL/mol of lysine. Thus, although 40 % of lysine residues were modified, only 2 % of these modifications could be accounted for by CEL, and only 1 % of the starting MGO was converted into CEL. In addition to the formation of MOLD at a yield comparable with that of CEL,

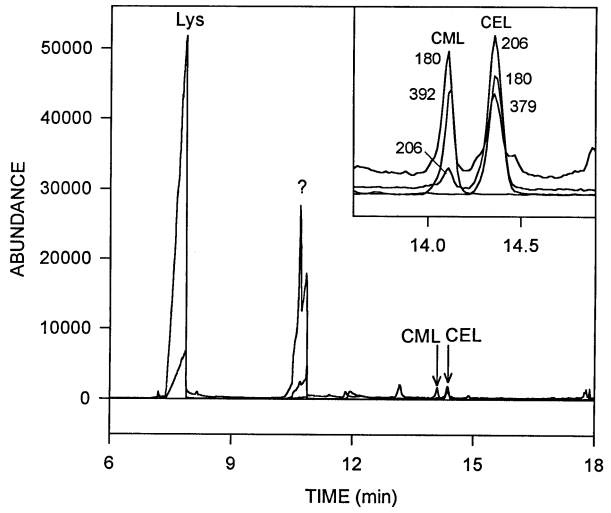

Figure 4 Detection of CML and CEL in lens protein by SIM-GC/MS

Lysine (*m/e* 180 and 320), CML (*m/e* 180, 305 and 392) and CEL (*m/e* 180, 206 and 379) were detected in lens (50-year-old donor) by their characteristic fragment ions [34].

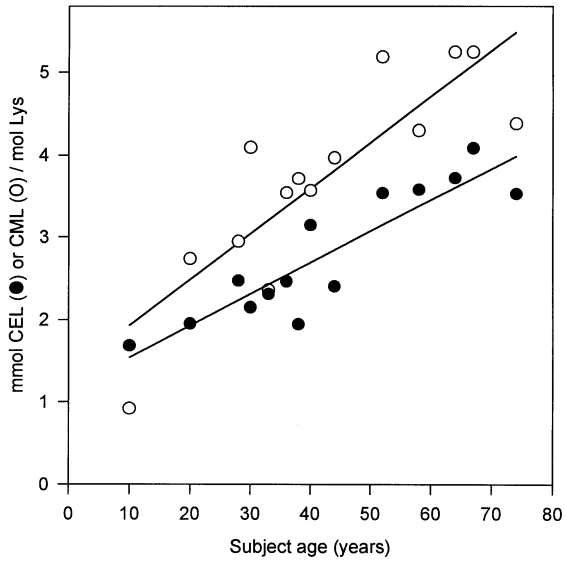

Figure 5 CML and CEL increase in concert in human lens proteins with age

There were statistically significant correlations between CML and age ($r = 0.85$, $P < 0.001$), between CEL and age ($r = 0.90$, $P < 0.001$), and between the concentrations of CML and CEL in lens proteins ($r = 0.80$, $P < 0.001$).

substantial browning, cross-linking and formation of fluorescent products occurred during the reaction, indicating that other, unidentified, products were also formed.

Measurement of CEL in human lens proteins

To assess the physiological relevance of reactions between MGO and protein, we analysed human lens proteins for CEL. These proteins, largely crystallins, were chosen because of their long biological half-lives and the fact that CML, a homologue of CEL, is known to accumulate in these proteins with age [33,34]. As shown in Figure 4, both CML and CEL were readily detectable in lens protein by SIM-GC/MS. Quantitative analysis by SIM-GC/MS (Figure 5) indicated that CEL, like CML, increased in lens proteins with donor age. The concentrations of CEL and CML in lens protein also correlated strongly with each another ($r = 0.80$, $P < 0.001$; see the legend to Figure 5). CEL was detected in human skin collagen at 5–10 % of the concentration detected in lens proteins from donors of similar age, and its concentration was approx. 8-fold higher in a pool of skin collagen samples from old, compared with young, donors (results not shown). These observations suggest that CEL, like CML, will be a useful biomarker of the chemical aging of tissue proteins.

We excluded the possibility that the compound identified as CEL in lens proteins could be the linear regioisomer N^{ε}-[1-(2-carboxy)ethyl]lysine (2-CEL). 2-CEL was synthesized by the reaction of equimolar amounts of hippuryl-lysine and acrylic acid methyl ester (50 mM) in methanol for 48 h at 65 °C. Following acid hydrolysis to remove the hippuryl group, 2-CEL was recovered in 40 % yield, based on amino acid analysis. 2-CEL was eluted between phenylalanine and lysine, compared with CEL which was eluted between valine and methionine, on our amino acid analyser system. 2-CEL was not detectable in reactions of MGO with N^{α}-acetyl-lysine (< 0.01 % yield). GC/MS analysis of the TFAME derivative of 2-CEL indicated that it eluted at 16 min, compared with 14.4 min for that of CEL. The

Table 1 Relative yields of CML and CEL on reaction of various carbohydrates with N^{α}-acetyl-lysine and collagen

With the exception of reactions between glucose and collagen, experiments were conducted for 96 h under oxidative conditions. Reactions with acetyl-lysine were conducted with equimolar (100 mM) concentrations of N^{α}-acetyl-lysine and carbohydrate. Collagen reactions contained 50–60 mg of collagen (wet weight) and 25 mM carbohydrate in 2 ml of phosphate buffer. Reactions of glucose with collagen contained 250 mM glucose and were conducted for 2 weeks. CML/CEL ratios are expressed as means ± range of two independent experiments conducted at different times. The absolute yields of CEL varied widely, from 0.001% (glucose) to 0.26% (MGO), for the various sugars.

	CML/CEL ratio	
Carbohydrate	N^{α}-Acetyl-lysine	Collagen
Glucose	175 ± 35	36 ± 4
Glucosone	215 ± 5	11.0 ± 0.5
3-Deoxyglucosone	76 ± 4	2.8 ± 0.1
Ascorbic acid	4.4 ± 1.0	2.1 ± 0.8
Ribose	17 ± 3	10.9 ± 1.0
Glyceraldehyde 3-phosphate	0.22 ± 0.01	0.1 ± 0.01
Dihydroxyacetone phosphate	0.23 ± 0.01	0.1 ± 0.01
MGO	0.09 ± 0.01	0.07 ± 0.01

mass spectrum of the TFAME derivative of 2-CEL yielded: 438 [M (the molecular ion), 1%], 406 ($M-CH_3OH$, 17%), 374 [$M-(CH_3OH)_2$, 8%], 347 ($M-CH_3OH-CH_3OCO$, 22%), 341 ($M-CF_3CO$, 32%), 180 ($M-CF_3CO-CH_3OCO-CH_3OCOCH_2CH_2N$, 40%), 69 (base peak, CF_3, 100%). The m/e 379 ion used for quantification of 2-CEL was present at only 2% relative intensity in CEL, and 2-CEL was not detectable in lens proteins (< 0.02 mmol/mol of Lys) by SIM-GC/MS using the m/e 406, 374, 347, 341 or 180 ions.

Origin of CML and CEL in tissue proteins

To identify possible sources of CML and CEL in tissue proteins, we investigated the formation of these compounds in reactions of a variety of carbohydrates with N^{α}-acetyl-lysine and rat tail collagen. These reactions were conducted for 4 days under the standard conditions used for the reaction of MGO with RNase, except that reactions with glucose were conducted for 2 weeks with 250 mM glucose to obtain sufficient products for analysis. The 4-day time period was chosen as a compromise, sufficient to allow for measurable formation of CML and CEL, although some substrates (MGO, ascorbate and glucosone) were consumed with a half-life of less than 1 day. All reactions were carried out under oxidative conditions, since oxidation is required for the formation of CML from hexose and pentose sugars and ascorbate.

As shown in Table 1, higher aldose sugars (glucose and ribose) and ascorbate yielded more CML than CEL, whereas MGO and triose phosphates were richer sources of CEL. Although similar trends were observed in reactions of the various carbohydrates with both N^{α}-acetyl-lysine and collagen, the CML/CEL ratios obtained in reactions with collagen were consistently lower than those obtained in reactions with N^{α}-acetyl-lysine. This may result from the reaction of arginine or other amino acids in collagen with glyoxal or MGO formed during autoxidation of carbohydrates, affecting the distribution of lysine adducts obtained. In some cases, intermediates in carbohydrate degradation might also be trapped by the protein, affecting the final distribution of CML and CEL. None of the carbohydrates tested yielded the approximate 1:1 ratio of CML/CEL observed in lens proteins (Figure 5), although the ratios obtained with 3-deoxyglucosone and ascorbate, two compounds that have been proposed as precursors or intermediates in the browning of lens proteins [36–38], were closest to the 1:1 ratio actually observed in lens proteins. These observations should be interpreted cautiously, however, because it is likely that different sugars, including glucose, ascorbate, triose phosphates and MGO, could contribute independently to the formation of CML and CEL in the lens.

DISCUSSION

Origins of CEL *in vivo*

In this study we have identified CEL as a stable chemical modification of proteins that is formed during non-enzymic Maillard reactions with MGO. We have also detected CEL in human lens proteins and shown that it accumulates in lens proteins with age. Krook et al. [39] have previously identified CEL as a non-stoichiometric modification of a specific lysine residue in $NADP^+$-dependent carbonyl reductase isolated from human placenta. These authors suggested that CEL might be formed enzymically by reduction of a Schiff-base adduct between pyruvate and lysine; however, non-enzymic formation from MGO was not excluded. Their observations suggest that carboxyethylation may be a highly site-specific chemical modification of proteins. Although the relationship between protein age and the CEL content of carbonyl reductase was not explored in the placental system, the formation of an acidic isoform of the enzyme is consistent with the age-dependent increase in anionic forms of enzymes in tissues [40].

Like CML, it is apparent that CEL is also formed non-enzymically from a variety of precursors (Table 1). CML may be produced from glyoxal formed on autoxidation of glucose [23], from glycolaldehyde formed on oxidation of Schiff-base adducts to proteins [24], or by oxidative cleavage of Amadori adducts on protein [25]. Recently, Fu et al. showed that CML is also formed during metal-catalysed oxidation of lipoproteins [41], probably from glyoxal, glycolaldehyde or other α-hydroxyaldehydes formed during lipid peroxidation reactions [42]. Thus, although CML is useful as an indicator of modification of proteins by products of carbohydrate or lipid autoxidation reactions, it is not a specific biomarker derived from a single precursor. Similarly, CEL may be formed not only from MGO, but also from triose phosphates and other sugars. Possible mechanisms for the formation of CEL from MGO include the Cannizzaro rearrangement [23] and sequential hydration and dehydration reactions [43,44]. It is uncertain whether the formation of CEL from 3-deoxyglucosone occurs by initial reaction of the dicarbonyl sugar with protein, followed by carbon–carbon bond cleavage, e.g. by a reverse aldol reaction, or by decomposition of the sugar to form MGO, which then reacts with protein to form CEL. The formation of CEL from triose phosphates could also proceed either by direct reaction of amino groups with the triose phosphate, followed by elimination of the phosphate group, or following spontaneous or amine-catalysed decomposition of triose phosphates to MGO. Studies on the CEL/CML ratios in different tissues and in tissues under various conditions, e.g. ascorbate deficiency or diabetes, may yield insight into the chemical origin of these carboxyalkyl derivatives of lysine residues in proteins.

Relationship between oxidative stress and concentrations of MGO and CEL in tissues

CML has been described as a glycoxidation product, because its formation from hexoses, pentoses or ascorbate and protein requires a combination of glycation and oxidation reactions [45].

Because of the role of both glycative and oxidative stresses in the formation of CML, changes in the levels of CML in tissue proteins [33,34,46] and urine [47] have been interpreted as a measure of the status of oxidative stress and of cumulative oxidative damage to proteins in aging and diabetes. Concentrations of CEL in tissue proteins may also be a useful biomarker and confirmatory indicator of the status of oxidative stress. Thus the concentration of MGO and other dicarbonyl sugars in tissues is a reflection of the efficiency of the glyoxalase and/or aldose reductase pathways in detoxifying dicarbonyl compounds. Increases in both substrates (MGO and acetol) and products (D-lactate and 1,2-propanediol) of these pathways in diabetes [7,15] suggest that the detoxification pathways may be overwhelmed by the flux of substrates. The efficiency of these pathways may also be compromised by decreased concentrations of the antioxidant glutathione, required for the glyoxalase pathway, and/or competition among various substrates, including glucose, for NADPH, a cofactor for aldose reductase. Decreases in glutathione concentrations have been noted in red cells from diabetic patients [48], in aortic endothelial cells from diabetic rabbits [49] and in the renal medulla of streptozotocin-diabetic rats [50], as well as in human endothelial cells [51] and rat lenses [52] cultured in hyperglycaemic media *in vitro*. Decreases in NADPH or shifts in the NADPH/$NADP^+$ ratio have also been noted in erythrocytes from diabetic patients [53], in the renal medulla of diabetic rats [50], and in human endothelial cells [51,54] and rat lenses [52] cultured in hyperglycaemic medium. These observations suggest that decreases in GSH and NADPH concentrations, and the resultant increased intracellular oxidative stress, may be a factor contributing to elevations in tissue MGO concentrations in diabetes. If increased chemical modification of proteins by MGO is involved in the increased browning and cross-linking of proteins in diabetes and the development of diabetic complications, then there should be an increase in the age-adjusted levels of CEL in tissue proteins and urine in diabetes, particularly in patients with complications. Continuing studies on tissue concentrations of MGO and CEL should yield a broader insight into the role of dicarbonyl compounds and oxidative stress in the chemical modification of proteins in aging and disease.

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