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CARBOHYDRATES, LIPIDS, AND OTHER NATURAL PRODUCTS:

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Carboxymethylethanolamine, a Biomarker of Phospholipid Modification during the Maillard Reaction *in Vivo**

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N^{ϵ}-(Carboxymethyl)lysine (CML) is a stable chemical modification of proteins formed from both carbohydrates and lipids during autoxidation reactions. We hypothesized that carboxymethyl lipids such as (carboxymethyl)phosphatidylethanolamine (carboxymethyl-PE) would also be formed in these reactions, and we therefore developed a gas chromatography-mass spectrometry assay for quantification of carboxymethylethanolamine (CME) following hydrolysis of phospholipids. *In vitro*, CME was formed during glycation of dioleoyl-PE under air and from linoleoylpalmitoyl-PE, but not from dioleoyl-PE, in the absence of glucose. *In vivo*, CME was detected in lipid extracts of red blood cell membranes, ~0.14 mmol of CME/mol of ethanolamine, from control and diabetic subjects, (n = 22, p > 0.5). Levels of CML in erythrocyte membrane proteins were ~0.2 mmol/mol of lysine for both control and diabetic subjects (p > 0.5). For this group of diabetic subjects there was no indication of increased oxidative modification of either lipid or protein components of red cell membranes. CME was also detected in fasting urine at 2–3 nmol/mg of creatinine in control and diabetic subjects (p = 0.085). CME inhibited detection of advanced glycation end product (AGE)-modified protein in a competitive enzyme-linked immunosorbent assay using an anti-AGE antibody previously shown to recognize CML, suggesting that carboxymethyl-PE may be a component of AGE lipids detected in AGE low density lipoprotein. Measurement of levels of CME in blood, tissues, and urine should be useful for assessing oxidative damage to membrane lipids during aging and in disease.

The nonenzymatic reaction of blood glucose with body proteins (glycation) followed by browning and oxidation reactions of glycated proteins leads to cumulative chemical modifications of tissue proteins throughout the body. These chemical changes, collectively termed the Maillard reaction, are considered to cause a gradual deterioration in the structure and function of tissue proteins and to contribute to the pathophysiology of normal aging (1–3). Further, the Maillard reaction is accelerated during hyperglycemia in diabetes, yielding advanced glycation end products (AGEs)[1] thought to be involved in the pathogenesis of diabetic complications (4–6). Among Maillard reaction products identified thus far in tissue proteins, concentrations of pentosidine (3, 7) and N^{ϵ}-(carboxymethyl)lysine (CML) (7) are known to increase in human skin collagen with age, and age-adjusted concentrations of both are increased in skin collagen in diabetes (7). Moreover, there is a strong relationship between levels of these products in collagen and the status of diabetic complications (8–10). Both CML and pentosidine require oxidative conditions for their formation, hence their description as glycoxidation products (11). Recently we showed that CML can also be formed during the reaction of autoxidizing polyunsaturated fatty acids (PUFA) with proteins (12), so that its precise biochemical origin is uncertain. The formation of CML during both glycoxidation and lipoxidation reactions emphasizes that Maillard chemistry is a general reflection of carbonyl amine reactions, whether the carbonyl compounds are derived from carbohydrates or lipids.

Several recent reports indicate that, like proteins, aminophospholipids are also targets of Maillard reactions. Pamplona *et al.* (13) first showed evidence for the presence of glycated phospholipids in rat liver and found their levels increased in animals with streptozotocin-induced diabetes. Ravandi *et al.* (14) further documented the existence of glycated aminophospholipids in human red blood cells and plasma, although their analytical technique did not distinguish between Schiff base and Amadori compounds, both of which are formed during the initial stage of the reaction of glucose with amino groups (1). Bucala *et al.* (15, 16) show *in vitro* that glycation of phosphatidylethanolamine (PE) but not phosphatidylcholine resulted in the formation of immunologically detectable AGEs in PE. In addition, using an ELISA assay, these authors concluded that the majority of AGEs present in low density lipoprotein isolated from normal and diabetic subjects

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[1] The abbreviations used are: AGE, advanced glycation end product; AGE-RbSA, AGE rabbit serum albumin; CM, carboxymethyl; CML, N^{ϵ}-(carboxymethyl)lysine; CME, carboxymethylethanolamine; PUFA, polyunsaturated fatty acids; PE, phosphatidylethanolamine; DO-PE, dioleoyl-PE; LP-PE, linoleoylpalmitoyl-PE; ELISA, enzyme-linked immunosorbent assay; GC/MS, gas chromatography-mass spectrometry; SIM-GC/MS, selected ion monitoring-GC/MS; PSR-1, anti-AGE protein antiserum; FL, fructose-lysine; TFAME, *N*,*O*-trifluoroacetyl methyl esters; BSA, bovine serum albumin.

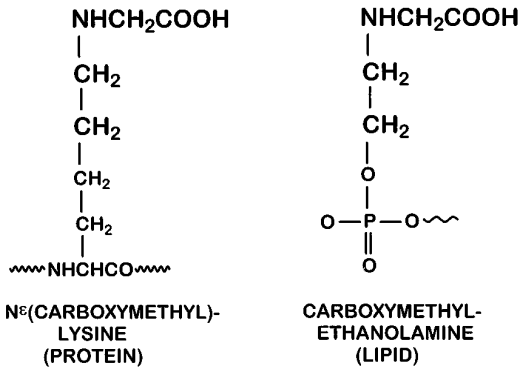


FIG. 1. **Structures of CML and CME in protein and lipid.** Enlarged portions emphasize the common features of each structure. The protein backbone is indicted by *compressed wave lines* and the diacylglyceride component of phospholipid is represented by *relaxed wave lines*.

were localized in the lipid phase (17). We recently identified CML as a major AGE antigen, and based on similarities in the structure of CML and CM-phospholipids such as CM-PE (Fig. 1), we proposed that AGE lipids may in fact, be immunologically cross-reactive carboxymethyl derivatives of aminophospholipids (18). Bucala *et al*. (15) also suggested that phospholipid glycation enhances lipid peroxidation. The interplay between glycation, glycoxidation, and lipid peroxidation may indeed be even more complex, since reaction of lipid decomposition products generated during peroxidative reactions with free amino groups of both proteins and aminophospholipids could lead to the formation of adducts chemically indistinguishable from carbohydrate-derived AGEs.

In the present study, we have developed a selected ion monitoring gas chromatography-mass spectrometry (SIM-GC/MS) assay for carboxymethylethanolamine (CME), the hydrolysis product of CM-PE, and present evidence for the presence of CME in human red cell membrane lipids and as a metabolite in urine. We also compare levels of CM protein and CM lipid in the red cell membrane and present their comparative levels in red blood cell ghosts in a group of control and diabetic individuals. Further, to gain insight into the nature of AGE lipids, we show that free CME can compete with protein-bound CML for recognition by an anti-AGE protein antiserum (PSR-1) previously shown to recognize CML as its primary epitope (18).

EXPERIMENTAL PROCEDURES

Materials—Glyoxylic acid and ethanolamine were purchased from Aldrich, d_4-ethanolamine was from Cambridge Isotope Laboratories (Andover, MA), and $NaBH_4$, $NaBH_3CN$, sarcosine, Dowex 50, dioleoylphosphatidylethanolamine (DO-PE), and linoleoylpalmitoylphosphatidylethanolamine (LP-PE) were from Sigma.

Synthesis and Isolation of CME and d_4-CME—Ethanolamine (0.33 mmol) and glyoxylic acid (1.33 mmol) were dissolved in 3 ml of phosphate buffer, pH 7.4. The mixture was heated at 65 °C for 1 h to allow Schiff base formation; solid $NaBH_3CN$ (0.33 mmol) was added, and the incubation was continued for an additional 2 h. Excess $NaBH_3CN$ was discharged by the addition of 1 ml of 6 N HCl, and the reaction mixture was dried *in vacuo*. Removal of borate as its methyl ester (19) was accompanied by the formation of a white precipitate of salts that was removed by centrifugation. After drying the supernatant *in vacuo*, the yellowish residue was dissolved in water, the pH was adjusted to 2.5 with formic acid, and the sample was applied to a 12-ml Dowex 50 cation exchange column equilibrated in dilute formic acid (pH 3). The column was washed with ~20 ml of the same formic acid solution and eluted with 0.5 N NH_4OH. Fractions (7 ml) were collected, the pH was measured, and the first 4 fractions after the pH became basic were pooled and dried. The sample was redissolved in 1% trifluoroacetic acid and applied to a 1-ml C-18 Sep-Pak column (Supelco Inc., Bellefonte, PA) equilibrated in the same solvent to remove brown impurities that were retained on the column. The column was washed with 2 volumes of 1% trifluoroacetic acid, and the eluates were combined and dried. The residue contained essentially pure CME, based on both amino acid and GC/MS analyses (see below). d_4-CME was prepared by the same procedure, using d_4-ethanolamine. Quantification of CME and d_4-CME standards was performed by cation exchange chromatography with postcolumn ninhydrin detection, using sarcosine as the primary standard.

Study Population—Patients with both insulin-dependent and noninsulin-dependent diabetes mellitus and control subjects were studied at the Medical University of South Carolina. The study was approved by the Institutional Review Board, and informed consent was obtained from all subjects. Control and diabetic groups were matched for age (26.6 ± 7.4 years, 26.7 ± 8.8 years, respectively), race (Caucasian/African American, 10/1), and gender (F/M, 6/5). Among diabetic subjects, the distribution of insulin-dependent to noninsulin-dependent diabetes mellitus was 9/2, and the average duration of diabetes was 8.3 ± 7.5 years. HbA1c values in control and diabetic blood were 5.7 ± 0.6% and 10.7 ± 2.7%, respectively ($p < 0.0001$). Patients were free of the vascular complications of diabetes, except for one patient who had both background retinopathy and microalbuminuria.

Isolation of Red Blood Cell Membrane Lipids and Proteins—Blood was collected into EDTA-containing Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), and all subsequent procedures were carried out at 4 °C. After centrifugation to separate plasma from red cells, red blood cell ghosts from ~8 ml of packed red cells were prepared by the method of Dodge *et al*. (20). Ghosts in 1 ml of 0.005 M phosphate buffer, pH 7.4, were supplemented with 1 mM diethylenetriaminepentaacetic acid and 0.01% butylated hydroxytoluene and then dried *in vacuo.* The membranes were extracted twice using chloroform/methanol (2:1); the lipid extracts were pooled, dried *in vacuo,* reduced with 100 mM $NaBH_4$ in methanol, and dried again. Ethanolamine and CME were measured in acid hydrolysates of the lipid extracts as described below. The extracted protein residues were dried and then acid-hydrolyzed, and CML and fructose-lysine (FL) were measured in the hydrolysates after derivatization as their *N*,*O*-trifluoroacetyl methyl esters (TFAME), all as described previously (19).

Analyses of Urine Samples—Second-voided fasting urine samples were obtained from all subjects and frozen at −70 °C until used. Aliquots containing ~100 μg of creatinine were mixed with 2 volumes of 0.2 M borate buffer, pH 9.2. $NaBH_4$ was added to a final concentration of 200 mM, and reduction was carried out overnight at room temperature. Excess borohydride was discharged by the addition of several drops of concentrated HCl, and borate was removed (19). After drying, deuterated internal standards were added, and samples were hydrolyzed in 6 N HCl for 24 h at 110 °C in screw cap test tubes that were purged with N_2. Hydrolysates were dried *in vacuo* and rehydrated in 1 ml of 1% trifluoroacetic acid, and brown impurities were removed using a 1-ml C-18 column as described above. The TFAME derivatives of the sample were prepared (19) and analyzed by SIM-GC/MS as described below. Creatinine was measured using a commercial kit (Sigma).

Glycoxidation and Autoxidation of PE—Incubations of PE with and without glucose were conducted in 0.2 M phosphate buffer, pH 7.4, for 2 weeks at 37 °C and were carried out under both oxidative and antioxidative conditions. For antioxidative conditions, samples in plain Vacutainer tubes were supplemented with 1 mM diethylenetriaminepentaacetic acid and 0.01% butylated hydroxytoluene, and the tubes were evacuated and flushed 3 × with nitrogen. For oxidative conditions, diethylenetriaminepentaacetic acid and butylated hydroxytoluene were omitted, and samples were opened every other day to allow entry of air. Lipids were extracted into chloroform/methanol (2:1), dried, and reduced with 50 mM $NaBH_4$ in 1 ml of methanol overnight at room temperature. Reduced lipids were dried *in vacuo* and, following the addition of deuterated internal standards, were hydrolyzed in 1 ml of 6N HCl for 24 h at 110 °C. After drying, the TFAME derivatives of the hydrolysates were prepared (19), and ethanolamine and CME were quantified by SIM-GC/MS as described below.

GC/MS Analyses—Analyses of TFAME derivatives of all samples except urine were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column coupled to a Hewlett-Packard model 6890 mass selective detector (Hewlett-Packard, Palo Alto, CA). The injection port was maintained at 275 °C. The temperature program was 60 °C to 115 °C at 2 °C/min, then ramp to 300 °C at 100 °C/min, and hold for 5 min. Quantification was by internal standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. SIM of 2 ions/analyte was performed, and similar results were obtained using both ions. The ions used were ethanolamine and d_4-ethanolamine, m/z = 140, 184 and 144, 188, respectively; CME and d_4-CME, m/z = 211, 266 and 214, 270, respectively (Fig. 2). Analyses of urine samples, which contained

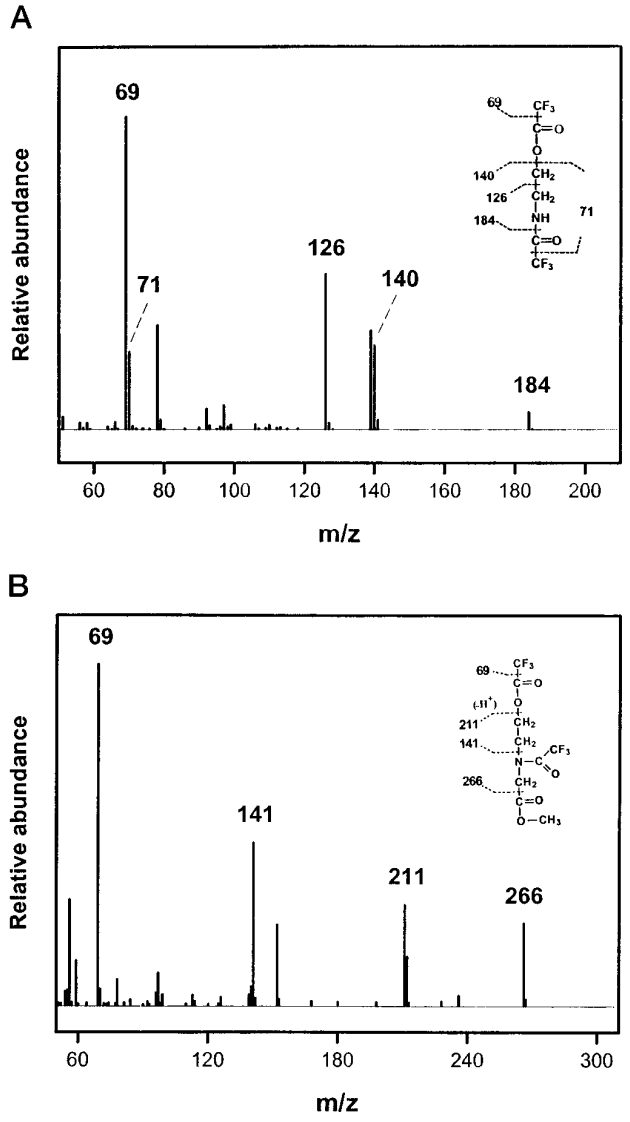
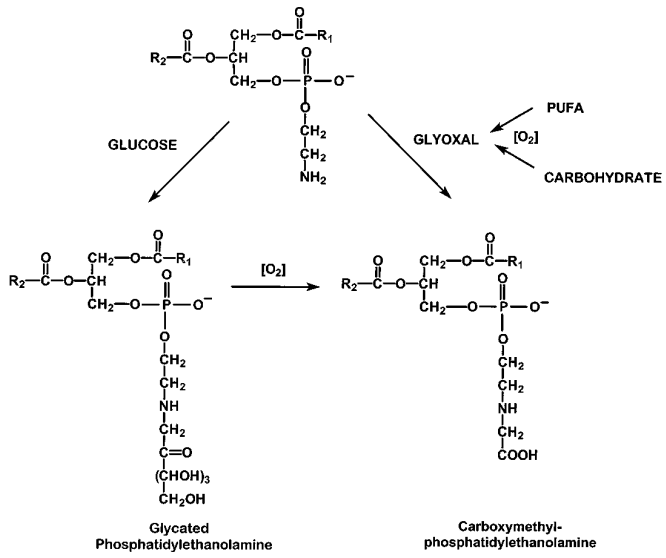


FIG. 2. **Full scan spectra and fragmentation patterns of ethanolamine and CME.** TFAME derivatives of authentic ethanolamine and synthetic CME are shown in *A* and *B*, respectively. The molecular ion of the CME (*m/z* = 325) was confirmed by chemical ionization mass spectrometry (data not shown).



SCHEME 1. **Possible routes to formation of CME in glycoxidation and/or lipoxidation reactions**.

numerous interfering substances, were carried out on a Hewlett-Packard model 5890 gas chromatograph equipped with a 30-m RTX 5 (5% phenyl) column (Restek Corp., Bellefonte, PA) coupled to a high resolution VG 70 SQ magnetic sector mass spectrometer (Fisons, Manchester, UK). The injection port was maintained at 250 °C. The temperature program was 2 min at 80 °C, ramp at 5 °C/min to 220 °C, ramp at 10 °C/min to 300 °C, and hold for 10 min. The same ions as listed above were monitored for measurement of ethanolamine and CME.

Immunochemical Assays—Competitive ELISA assays were performed by the method of Reddy *et al.* (18) with minor modifications. Briefly, 96-well polystyrene plates coated with AGE rabbit serum albumin (AGE-RbSA) (20 ng/well) were incubated with mixtures of competing antigens and a 1:5000 dilution of rabbit antiserum PSR-1. Antibody binding was detected using peroxidase-conjugated anti-rabbit IgG as second antibody.

Statistical Analysis—All data are expressed as mean ± S.D., and significance was assessed by Mann-Whitney nonparametric analysis.

RESULTS

Characterization of CME Formation on PE by Lipoxidaton or Glycoxidation—CME may be formed on PE by at least three routes (Scheme 1), including glycation of PE and oxidative cleavage of the Amadori adduct or from the reaction of glyoxal formed by autoxidation of glucose or PUFA. CME was produced during autoxidation of PL-PE in phosphate buffer (Fig. 3*A*, Table I) but not under antioxidative conditions. These results are consistent with the formation of glyoxal during PUFA autoxidation (12, 21, 22). CME was also formed in incubations of DO-PE with glucose (Fig. 3*B*, Table I) but not from DO-PE incubated in buffer only. Although the glucose levels in these incubations are clearly nonphysiological, the reaction conditions were modeled on those of Bucala *et al.* (15) because of the detection of AGE lipid in this system. CME was also produced, but at lower yield (10–15%), in antioxidative incubations of DO-PE with glucose (Table I). This finding was consistent in three separate experiments. Both oxidative and antioxidative incubations of DO-PE in the presence of glucose developed a brown color with time, indicating that even in the absence of PUFA, aminophospholipids are active substrates for the Maillard reaction in aqueous systems. LP-PE developed a light yellow color during 2 weeks of incubation under air.

The amounts of CME formed after 2 weeks of incubation of various reaction mixtures are summarized in Table I. Whereas the concentration of LP-PE was only 10% that of DO-PE, for reasons of economy, the amount of CME formed under oxidative conditions in LP-PE was about half that of DO-PE incubated with glucose. Thus, autoxidation of PE containing unsaturated PUFA was an efficient source of CME. The CME detected in samples prepared *in vitro* and from *in vivo* sources (see below) was measured after reduction with $NaBH_4$ so that its formation as an artifact from either carbohydrate or lipid adducts during acid hydrolysis is unlikely.

Quantification of CME, CML, and FL in Red Cell Ghosts—As shown in Fig. 4, CME was readily detected in hydrolysates of red cell membrane lipid extracts. Levels of CME in erythrocyte membrane lipids from control subjects and diabetic patients are compared in Fig. 5. CME values were, respectively, 0.135 ± 0.054 and 0.146 ± 0.081 mmol/mol of ethanolamine; these data were calculated excluding the two high CME values shown in parentheses because they were >2 S.D. above the mean. The mean CME values were not significantly different using nonparametric, Mann-Whitney analysis. For comparison, results of analyses of CML and the Amadori compound, FL, on membrane protein fractions from these same samples are also shown in Fig. 5. CML concentrations in control and diabetic samples were 0.213 ± 0.063 and 0.184 ± 0.062 mmol/mol of lysine, respectively, and were also not significantly different. Of note, the ratio of CME/ethanolamine in the lipid fraction

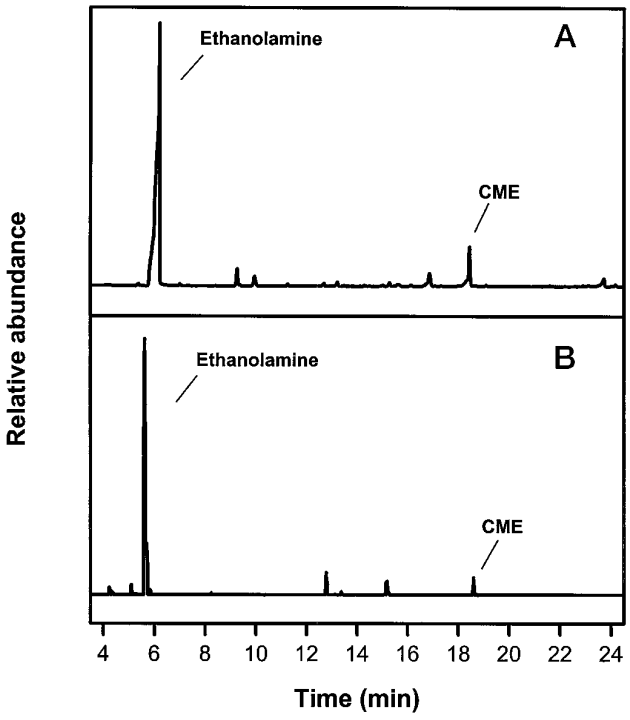


FIG. 3. **Formation of CME during lipoxidation and glycoxidation of PE.** *Traces* shown are SIM chromatograms of hydrolysates from phospholipid incubations carried out at 37 °C for 2 weeks under aerobic conditions. *A*, LP-PE (1.4 mM) incubated in 0.5 M phosphate buffer, pH 7.4; *B*, DO-PE (14 mM) incubated in buffer with 500 mM glucose. No CME was present in either original phospholipid preparation. Retention times and mass spectra of ethanolamine and CME were the same as those obtained for TFAME derivatives of authentic CME and ethanolamine shown in Fig. 2.

TABLE I
Recovery of CME in PE incubated under oxidative and antioxidative conditions

PE samples were incubated for 2 weeks in 0.2 M phosphate buffer, pH 7.4, at 37 °C, and the concentrations of CME and ethanolamine were determined as described under "Experimental Procedures."

Phospholipid	Oxidative	Antioxidative
	mmol of CME/mol of ethanolamine	
DO-PE (14 mM)	0	
DO-PE (14 mM) + glucose (500 mM)	17.7	2.7
LP-PE (1.4 mM)	8.7	0

was similar to the ratio of CML/lysine in the protein fraction, suggesting an overall similar extent of carboxymethylation of aminophospholipids and proteins in red cell membranes. At the same time, FL values, reflecting both ambient glucose concentration and steady state level of protein glycation (1), were elevated in a statistically significant fashion in diabetic (5.27 ± 1.42 mmol/mol of lysine) *versus* control samples (2.13 ± 1.33 mmol/mol of lysine, $p < 0.01$). The approximately 2.5-fold increase in mean FL concentration is comparable to the approximate 2-fold increase in mean HbA1c values, and there was an overall significant correlation between FL and HbA1c values ($r = 0.54$, $p = 0.005$).

Measurement of CME and CML in Human Urine—As shown in Fig. 6, the mean concentrations of CM species in normal urine were 1.82 ± 0.70 nmol of CME/mg of creatinine and 5.58 ± 2.11 nmol of CML/mg of creatinine, respectively, corresponding to a CML/CME ratio of 2.6. CME is present in urine primarily in its free form, *i.e.* as the product of hydrolysis of CM-PE (data not shown), suggesting active catabolism of the latter compound. CME and CML concentrations in the urine of

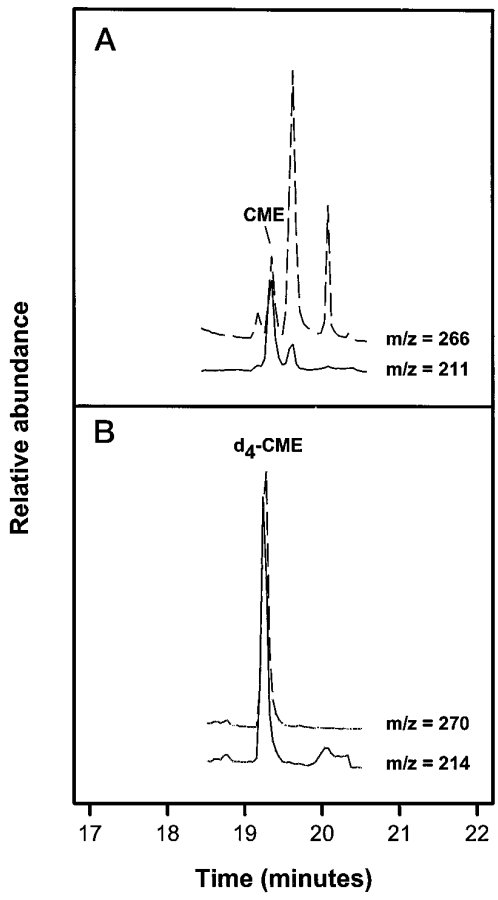


FIG. 4. **Detection of CME in a human red cell membrane lipid extract.** Selected ion chromatograms are shown for characteristic CME ions in a lipid extract from control subject (*A*) and internal standard d_4-CME (*B*).

diabetic patients were 2.92 ± 1.76 nmol of CME/mg of creatinine and 8.53 ± 4.96 nmol of CML/mg of creatinine, respectively. Although the means were somewhat higher than for the control population, they did not reach statistical significance. In a previous study, using a larger number of samples, we reported a modest, though significant elevation of urinary CML in diabetes (19). In the present study there was, however, a strong correlation between urinary CME and CML ($r = 0.737$, $p = 0.0003$, $n = 9$).

Immunochemical Detection of CME by Anti-AGE Protein Antibody—We have previously shown that CML-BSA is a potent competitor for the recognition of AGE-RbSA by antiserum PSR-1, demonstrating that CML is a dominant AGE antigen (18). CML as the free amino acid is a significantly weaker competitor, about 10^3–10^4 times less efficient than CML-BSA. As shown in Fig. 7, CME was comparable to CML in inhibiting the recognition of AGE-RbSA by the PSR-1 antiserum. Ethanolamine, like lysine (not shown), was completely ineffective.

DISCUSSION

In this study we describe the detection and measurement of a CM lipid, CME, in human membrane lipids and urine. Based on the concentration of CME in ghost membranes, ~0.14 mmol/mol of PE (Fig. 5), and the concentration of PE in red cell membranes, ~1 mg/ml of packed red cells (23) (average molecular mass for PE, 800 Da), there is an estimated 0.18 nmol of CME/ml of packed erythrocytes. From the recovery of ~2 nmol of CME/mg of creatinine (Fig. 6) and a mean daily excretion of 1.4 g of creatinine for a 70-kg subject, about 2.8 μmol (0.3 mg of CME) are excreted daily by a healthy human adult. Since the

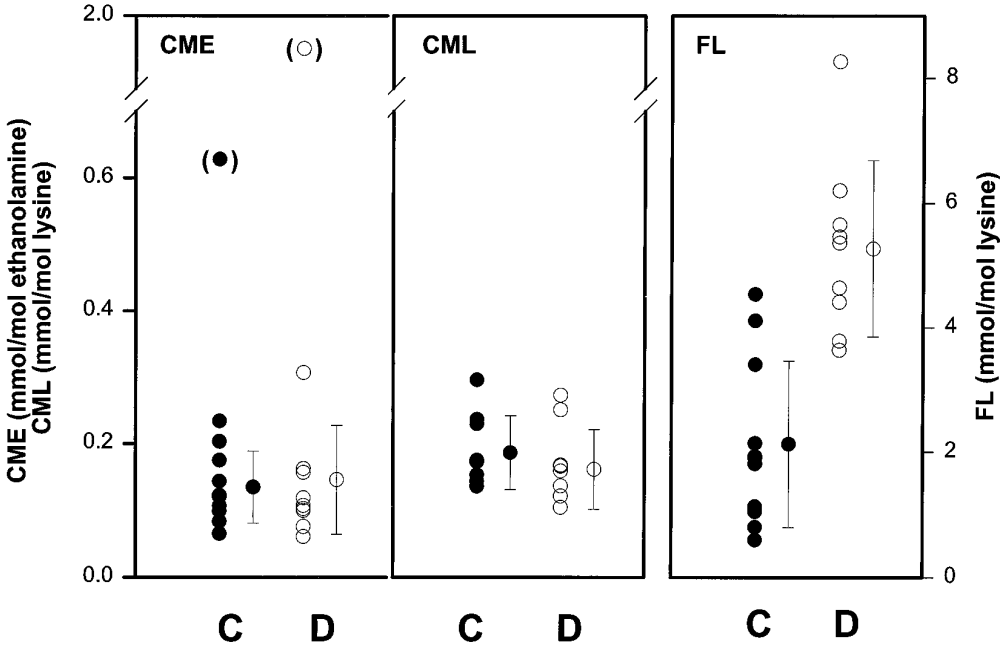


FIG. 5. **Comparison of CME, CML, and FL in human red blood cell membrane lipids and proteins.** CME, CML, and FL were analyzed as described under "Experimental Procedures." *C*, control subjects; *D*, diabetic patients. $n = 11$ for FL and CME analyses; $n = 9$ and 8 for CML in control and diabetic subjects, respectively.

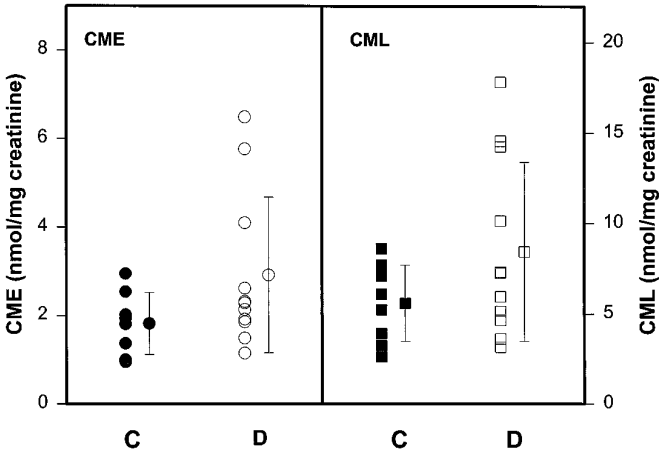


FIG. 6. **Levels of CME and CML in human urine.** Fasting urine was collected from the same subjects shown in Fig. 5. CME and CML were analyzed as described under "Experimental Procedures." *C*, control; *D*, diabetic subject. $n = 11$ for all analyses except $n = 9$ for control CME.

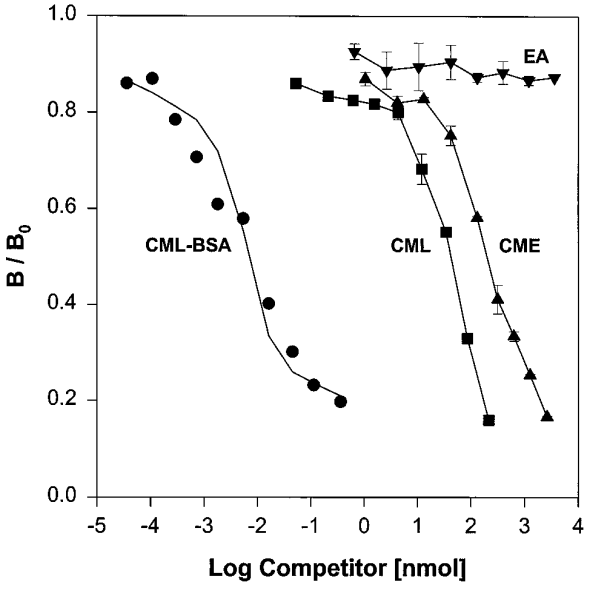


FIG. 7. **Immunochemical detection of CME by anti-AGE protein antibody.** Competitive ELISA assays were performed after coating wells with AGE-RbSA and the addition of anti-AGE-protein antibody, PSR-1, with indicated amounts of competing antigens. *EA*, ethanolamine. Data are expressed as the ratio of absorbance of sample (*B*) to absorbance in sample incubated in the absence of competitor (B_0) after background subtraction.

average 70-kg subject has about 2 liters of packed red cells and ~1% of red cells turnover/day (average red cell life span, 120 days), only about 4 nmol of the daily urinary excretion of CME can be attributed to red cell membrane degradation. This estimate emphasizes that >99% of the 2.8 μmol of CME recovered daily in urine arises from the normal turnover of membrane phospholipids in cells other than red cells. Thus, CME represents a natural metabolite formed during glycoxidative and/or lipoxidative modification of phospholipids, and changes in these levels in urine may reflect changes in overall oxidative stress *in vivo*. CM-serine has also been detected in hydrolysates of ghost lipids and in urine (data not shown), indicating that carboxymethylation of both PE and phosphatidylserine occurs under physiological conditions.

Origins of CME—Our model reactions show that CME can be formed from either glucose or PUFA under oxidizing conditions. It is difficult to make a direct comparison of the relative efficiency of each substrate in forming CME. Thus, in *in vitro* experiments (Table I), glucose, which is resistant to autoxidation compared with PUFA, was present at a 33-fold molar excess over free amino groups, whereas the PUFA (linoleic acid) in LP-PE is present at the same concentration as the amino group. Since aminophospholipids form micelles in aqueous buffers, it is likely that reactive intermediates formed during peroxidation of PUFA *in situ* may be present at very high local concentrations. At this point we can only speculate about the metabolic origin of CM-PE. It is difficult to anticipate which of the routes, glycoxidation, glucose, or PUFA autoxidation, shown in Scheme 1 predominates *in vivo,* and thus CME, like CML, may be both a glycoxidation and lipoxidation prod-

uct. Jain *et al*. (24, 25) previously reported a serine-malondialdehyde-ethanolamine cross-link in red cell membranes, which is another nonenzymatically modified aminophospholipid found *in vivo,* but in this case the origin of the modification from lipid peroxidation is more certain. This cross-link structure was reported to be elevated approximately 3-fold in diabetic subjects (24), but the complication status of the subjects was not described.

Physiological Significance of CME—CME is quantitatively a minor fraction of total ethanolamine, representing about only 0.01% modification of red blood cell membrane phospholipid. It can be estimated that there are $\sim 10^4$ molecules of CME/red cell. Thus, there are $\sim 10^{10}$ red cells and 0.18 nmol of CME (see above) or $\sim 10^{14}$ molecules of CME/ml of packed red cells, yielding a value of $\sim 10^4$ molecules of CME/red cell. Anticipating that serine will be modified to a similar extent, it seems likely that CM lipids, like CML, are biomarkers rather than important effectors of damage in tissue. At the same time, AGEs are hypothesized to play a key role as causative agents in the pathogenesis of diabetic complications, and in agreement with this notion, CML and other AGEs are elevated in lens and skin collagen of diabetic patients and correlate with severity of complications (8–10, 26). Since the patients participating in this study were free of complications, it is not surprising that there were not differences in markers of oxidative damage between control and patient populations. Studies are in progress to examine levels of CM lipids in diabetic patients with more extensive vascular complications who are predicted to show differences in the levels of both carboxymethylated lipids and protein. Finally, consistent with the red cell data, neither CME or CML concentrations were significantly elevated in the urine of the diabetic subjects in this study. The modest but statistically insignificant increase in urinary CM species is more than sufficiently explained by the larger increases in the amounts of oxidizable substrates (glucose, glycated proteins and glycated aminophospholipids, and triglycerides) without the necessity of invoking a diabetes-dependent increase in oxidative stress.

Although PE and phosphatidylserine are located on the inner layer of the red cell membrane bilayer, they become externalized under a variety of pathological conditions, including oxidative stress to red cells (27, 28). Should this also be the case for CM lipids (and/or CML); once exposed on the outer membrane of erythrocytes, they may have a role in the recognition by and binding to receptors for AGEs reported on a variety of cell types, including endothelial cells and macrophages. Wautier *et al*. (29) describe the interaction of erythrocytes from diabetic rats with one such receptor termed RAGE. According to these authors, this interaction could contribute to the shortened half-life of red blood cells isolated from diabetic rats when injected into normal rats and also lead to a localized increase in oxidative stress in the vascular wall, ultimately contributing to the pathogenesis of diabetic vascular disease. The AGE structure(s) on the surfaces of red cells that mediate their interaction with the RAGE has not been characterized. It seems possible that CM lipids (and CML) measured in the present study could form part of an array of negative charge that may be recognized by RAGE or other scavenger receptors involved in erythrocyte turnover.

CME as an AGE Antigen—Because the terminal $-CH_2CH_2NHCH_2COOH$ moieties of CM-lysine and the predicted structure of CM-PE (Fig. 1) are identical, it seemed likely that anti-AGE protein antibodies might also recognize CM lipids. Our results indicate that CME is an effective competitor for AGE protein binding to PSR-1, an anti-AGE protein antiserum we have shown to recognize CML as a major epitope (18), emphasizing that CM phospholipids in lipoproteins may contribute to the AGE content of serum measured by ELISA. Although we do not know how much better the recognition of CM-PE in membrane or other lipoproteins may be, by analogy to the results obtained for free CML and CML-BSA, CM-PE may be an important epitope recognized by anti-AGE protein antisera. Indeed, some of the AGE lipids described in low density lipoprotein by Bucala *et al*. (15, 16) may be CM-aminophospholipids. Although the antibody used in their analyses was reported to be unreactive toward CML (17), present knowledge suggests that, given the significantly lower reactivity of free CML compared with protein-bound CML to anti-AGE protein antibody (18, Fig. 6), the amount of competing free CML used in their experiments may not have been sufficient to yield effective competition. At the same time, it should be noted that these workers reported a 4-fold increase of AGE lipids in low density lipoprotein from diabetic patients *versus* healthy subjects (15, 16), suggesting a greater increase in Maillard modification of aminophospholipids in diabetes than indicated by our results. The differences between our results may reflect differences in the severity of complications in the patient groups. Finally, Kume *et al.* (30), using a monoclonal antibody to AGE-protein, 6D12, reported the presence of AGEs in frozen sections prepared from atherosclerotic tissue of normoglycemic humans. This antibody has since been shown to recognize CML (31); however, it is possible that both CM protein and lipid may have been detected in this study.

In summary, we describe the detection of CM-PE *in vivo* and present quantitative data on its concentration in human red blood cell membrane lipids and on the concentration of its metabolite, CME, in urine. We also demonstrate that CME may be formed by autoxidation of both carbohydrate and lipid. Measurements of CME in different tissues should prove useful for assessing the role of glycoxidation and lipoxidation or, more generally, carbonyl stress (32) in the pathogenesis of diabetes, its complications, and other disease processes.

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