

LC-MS/MS analysis of carboxymethylated and carboxyethylated phosphatidylethanolamines in human erythrocytes and blood plasma[§]

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Abstract An amino group of phosphatidylethanolamine (PE) is considered as a target for nonenzymatic glycation, and the potential involvement of lipid glycation in the pathogenesis of diabetic complications has generated interest. However, unlike an early glycation product of PE (Amadori-PE), the occurrence and roles of advanced glycation end products of PE (AGE-PE) in vivo have been unclear. Here, we developed an LC-MS/MS method for the analysis of AGE-PE [carboxymethyl-PE (CM-PE) and carboxyethyl-PE (CE-PE)]. Collision-induced dissociation of CM-PE and CE-PE produced characteristic ions, permitting neutral loss scanning (NLS) and multiple reaction monitoring (MRM) of AGE-PE. By NLS analysis, a series of AGE-PE molecular species was detected in human erythrocytes and blood plasma. In LC-MS/MS analysis, MRM enabled the separation and determination of the predominant AGE-PE species. Between healthy subjects and diabetic patients, no significant differences were observed in AGE-PE concentrations in erythrocytes and plasma, whereas Amadori-PE concentrations were higher in diabetic patients. These results provide direct evidence for the presence of AGE-PE in human blood, and indicated that, compared with Amadori-PE, AGE-PE is less likely to be accumulated in diabetic blood. **¶** The presently developed LC-MS/MS method appears to be a powerful tool for understanding in vivo lipid glycation and its pathophysiological consequence.—Shoji, N., K. Nakagawa, A. Asai, I. Fujita, A. Hashiura, Y. Nakajima, S. Oikawa, and T. Miyazawa. **LC-MS/MS analysis of carboxymethylated and carboxyethylated phosphatidylethanolamines in human erythrocytes and blood plasma.** *J. Lipid Res.* 51: 2445–2453.

Supplementary key words lipid glycation • advanced glycation end products • tandem mass spectrometry • diabetes

Nonenzymatic protein glycation and its involvement in diabetic complications have been thoroughly investigated (1). In addition to the protein glycation, we and some researchers have found that glycation reaction also occurs in vivo between lipids and glucose (2–4). It suggests that membrane phospholipids such as phosphatidylethanolamine (PE) are abnormally glycated under hyperglycemic conditions and that lipid glycation may contribute to the pathogenesis of diabetic complications (e.g., retinopathy, nephropathy, neuropathy, and atherosclerotic macrovascular disease). We previously investigated the pathophysiological impact of lipid glycation in vitro and found that Amadori-PE (an early glycation product of PE,; **Fig. 1A**) caused lipid peroxidation (5) and angiogenesis (6). We also developed a sensitive assay for Amadori-PE and demonstrated its accumulation in blood plasma of diabetic patients (7). Knowledge gained from these studies (2–7) provides insight into the involvement of Amadori-PE in the pathogenesis of diabetic complications (8, 9).

Once Amadori-PE is formed, it can further undergo complex reactions to form PE-linked advanced glycation end products (AGE-PE) such as carboxymethyl-PE (CM-PE) and carboxyethyl-PE (CE-PE) (**Fig. 1B**) (10, 11). Similarly to Amadori-PE, AGE-PE is also hypothesized to trigger pathological processes (10, 11); however, to our knowledge, there are only three reports investigating the presence of AGE-PE in vivo (12–14). Requena et al. (12) and Pamplona et al. (13) demonstrated the presence of CM-PE

Abbreviations: AGE, advanced glycation end product; CE, carboxyethyl; CM, carboxymethyl; PE, phosphatidylethanolamine; MRM, multiple reaction monitoring; NLS, neutral loss scanning.

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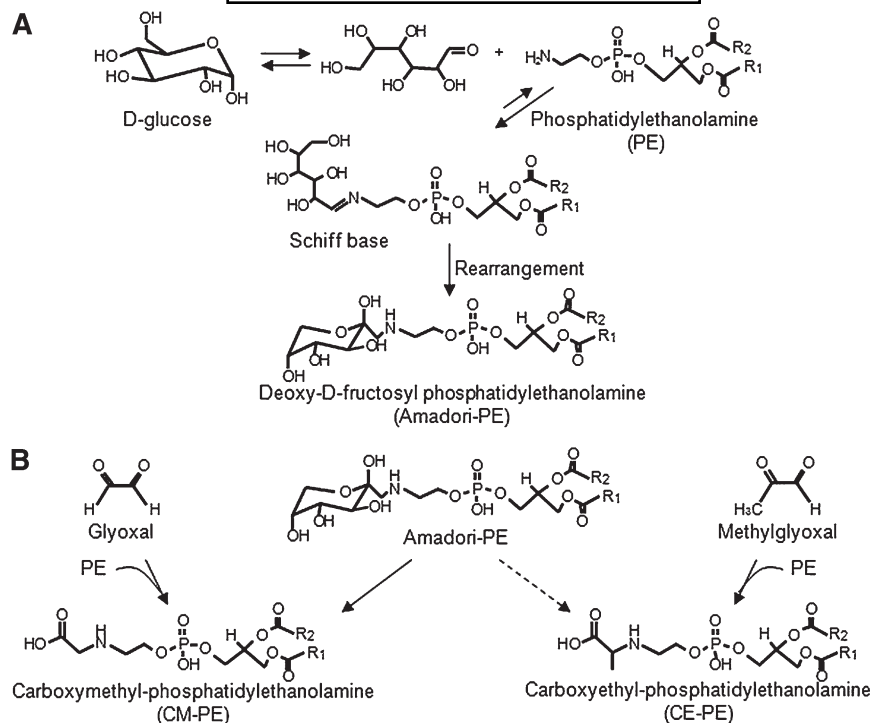


Fig. 1. Scheme for the glycation of PE. A: Glucose reacts with the amino group of PE to form a Schiff base, which undergoes an Amadori rearrangement to yield the Amadori-PE (deoxy-D-fructosyl PE). B: Possible routes to formation of CM-PE and CE-PE in the late stage of glycation.

in human erythrocytes and mammalian mitochondrial membranes, respectively. In contrast, Breitling-Utzmann et al. (14) reported that neither CM-PE nor CE-PE was detected in blood samples. Because of the potential consequence of AGE-PE in diabetic complications, these conflicting results imply the need for a new analytical mean to accurately measure CM-PE and CE-PE. To address this need, we aimed to develop a quantitative method to analyze CM-PE and CE-PE by using LC-MS/MS. With the developed method, we analyzed CM-PE and CE-PE in erythrocyte and plasma of healthy subjects and diabetic patients to assess the effects of hyperglycemia on the accumulation of early and advanced glycation end products of PE (Amadori-PE and AGE-PE, respectively).

EXPERIMENTAL PROCEDURES

Materials

All PE molecular species were purchased from Avanti Polar Lipids (Alabaster, AL). The molecular species were indicated by the carbon chain length and the unsaturation degree of the *sn*-1,2 acyl chains; e.g., "16:0-18:1 PE" for 1-hexadecanoyl-2-octadecenoyl-*sn*-glycero-3-phosphoethanolamine. CM-PE and CE-PE standards were synthesized by using the PE species as starting materials, as described by Utzmann and Lederer (15), with modifications. In brief, for the synthesis of CM-PE, PE (10 μ mol), glyoxylate (100 μ mol), and cyanoborohydride (10 μ mol) were dissolved in 100 ml of methanol and incubated at 60°C for 4 h. For the synthesis of CE-PE, pyruvate (100 μ mol) was used instead of glyoxylate. The reaction mixture was evaporated to dryness, and the residue was dissolved in 10 ml of chloroform-methanol

(2:1, v/v). CM-PE and CE-PE were then isolated by using preparative LC. Under the present conditions, for instance, 5 mg of pure 18:1-18:1 CM-PE (>99% by LC-MS analysis) was yielded from 7 mg of 18:1-18:1 PE. Amadori-PE was prepared as we reported previously (5). The structure and purity of each synthesized compound were evaluated by LC-MS (Mariner, Applied Biosystems, Foster City, CA), high resolution fast atom bombardment (FAB)-MS (JEOL-JMS-700 mass station, JEOL, Tokyo, Japan), and NMR (Varian Unity 600 spectrometer, Varian, Palo Alto, CA). All other reagents were of the highest grade available.

In vitro glycation of 18:1-18:1 PE

18:1-18:1 PE (9 μ mol) was incubated with glucose (15 mmol) in 30 ml of 0.1 M phosphate buffer-methanol (3:7, v/v, pH 7.4) at 60°C. A portion of the reaction mixture was collected at 0, 3, 6, 24, and 48 h, and the lipid-soluble products were extracted with chloroform-methanol (2:1, v/v) (16).

Blood sample preparation

Human blood samples were collected from eight healthy volunteers (four females and four males, age 22 \pm 1 years) and 10 newly detected type 2 diabetic patients (seven females and three males, age 55 \pm 12 years, hemoglobin A1c 12 \pm 2%). None of the diabetic patients received any medication for the control of blood glucose at the time of blood collection. This study was approved by the institutional review board of the Nippon Medical School, Tokyo, Japan, and informed consent was obtained from all participants.

Blood (10 ml) was collected into a tube containing EDTA-2Na as an anticoagulant and centrifuged at 1,000 g for 10 min at 4°C. After plasma and buffy coat were removed, erythrocytes were washed three times with phosphate-buffered saline (pH 7.4) to prepare packed cells. Total lipids were extracted from 1 ml of the packed cells using 2-propanol and chloroform (17). Plasma (1 ml) was subjected to total lipid extraction using Folch's partition

(16). Each extract was evaporated to dryness, and the residue was dissolved in 1 ml of chloroform-methanol (2:1, v/v).

MS/MS analysis

A 4000 QTRAP quadrupole/linear ion-trap tandem mass spectrometer (Applied Biosystems) was used for MS/MS analysis. Initially, we analyzed synthesized 18:1-18:1 CM-PE and 18:1-18:1 CE-PE as the reference compounds of AGE-PE. To evaluate the MS/MS fragmentation, product ion scanning was performed by directly injecting 18:1-18:1 CM-PE or 18:1-18:1 CE-PE solution (0.2 nmol/ml methanol) into MS/MS (5 μ l/injection) using methanol as carrier solvent (0.2 ml/min). Electrospray ionization was used as an ion source with collision energy of 33 eV, transition dwell time of 100 ms, turbo gas temperature at 500°C, and spray voltage of 5,000 V. Nitrogen pressure values for turbo, nebulizer, and curtain gases were set at 30, 60, and 10 pounds per square inch, respectively. Positive ion spectra were collected in the m/z range of 100–900.

Neutral loss scanning (NLS) was performed for profiling CM-PE and CE-PE molecular species in blood samples. The MS/MS instrument was programmed to scan parent ions that yielded a neutral loss of 199 Da for CM-PE or of 213 Da for CE-PE after fragmentation in the collision cell. Sample (erythrocyte extract or plasma extract, 5 μ l each) was injected directly into MS/MS as described above. Amadori-PE and PE molecular species were also evaluated using NLS of 303 Da and 141 Da, respectively.

LC-MS/MS analysis with multiple reaction monitoring

For LC-MS/MS analysis, a Shimadzu liquid chromatography system, including a vacuum degasser, a quaternary pump, and an autosampler (Shimadzu, Kyoto, Japan), was equipped with the 4000 QTRAP mass spectrometer. CM-PE and CE-PE were analyzed using an ODS column (Xbridge, 2.1 \times 100 mm; Waters, Tokyo, Japan) with an isocratic mobile phase of methanol-water

(99:1, v/v; containing 5 mM ammonium acetate). The flow rate was set at 0.2 ml/min, and the column temperature was maintained at 40°C. CM-PE and CE-PE were detected using multiple reaction monitoring (MRM) for the transition of parent ions to product ions. For the quantitation of CM-PE and CE-PE in blood samples, we focused on eight molecular species (16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6) of both CM-PE and CE-PE, because their presence in erythrocytes and plasma was revealed by NLS in MS/MS analysis. Using synthesized AGE-PE species, we prepared standard solutions at concentrations of 5–1,000 pmol/ml (a range expected to encompass concentrations encountered in vivo). The erythrocyte extract, plasma extract, or the standard solution (2 μ l each) was then subjected to LC-MS/MS, and the AGE-PE molecular species were individually detected using MRM. The concentrations of CM-PE and CE-PE in erythrocytes and plasma were calculated using the calibration curves of the synthesized AGE-PE molecules.

The MRM detection was also applied for Amadori-PE and PE. Amadori-PE and PE were analyzed using a silica column (Inertsil, 2.1 \times 100 mm; GL Sciences, Tokyo, Japan) with a binary gradient consisting of solvent A [acetonitrile-methanol-1 M aqueous ammonium formate (pH 6.0) (78:20:2, v/v/v)] and solvent B [acetonitrile-methanol-1 M aqueous ammonium formate (pH 6.0) (49:49:2, v/v/v)]. The gradient profile was as follows: 0–1.25 min, 10% B; 1.25–2 min, 10–100% B linear gradient; 2–6 min, 100% B. The flow rate was 0.4 ml/min, and the column temperature was at 40°C. The concentrations were calculated using the calibration curves of synthesized Amadori-PE and authentic PE.

Statistics

The data are expressed as means \pm SD and analyzed using Student's *t*-test. Differences were considered significant at $P < 0.01$.

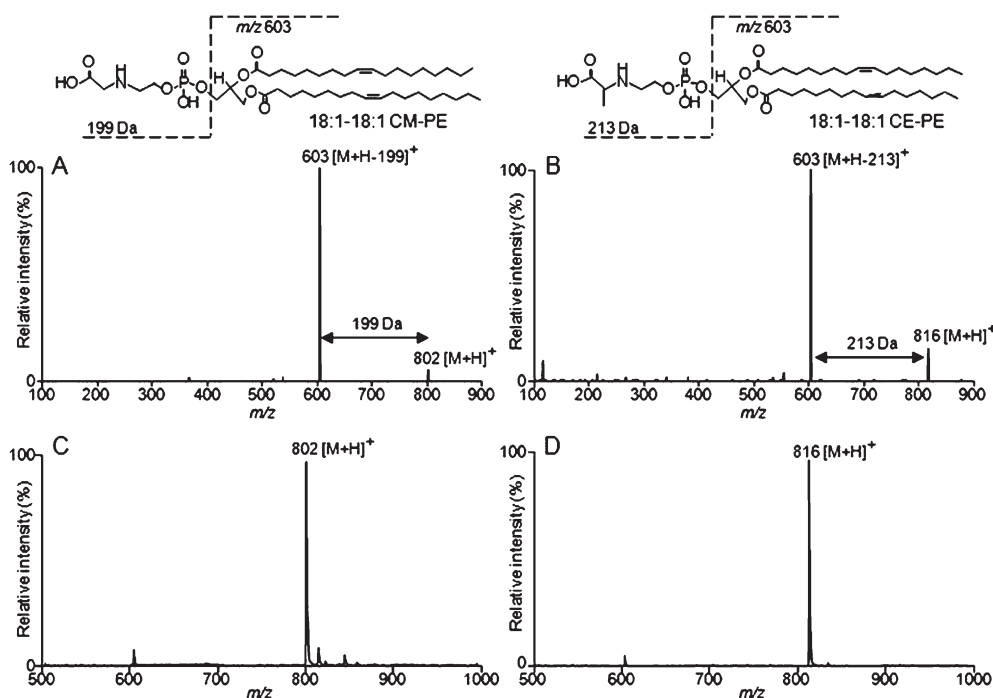


Fig. 2. MS/MS analysis of CM-PE and CE-PE. Product ion spectrum of the $[M+H]^+$ ion at m/z 802 of 18:1-18:1 CM-PE (A) and m/z 816 of 18:1-18:1 CE-PE (B). NLS of 199 Da and 213 Da showing specific detection of 18:1-18:1 CM-PE (C) and 18:1-18:1 CE-PE (D), respectively. The synthesized 18:1-18:1 CM-PE or 18:1-18:1 CE-PE (each 1 pmol) was injected directly into MS/MS by an LC pump at the eluent (methanol) flow rate of 0.2 ml/min.

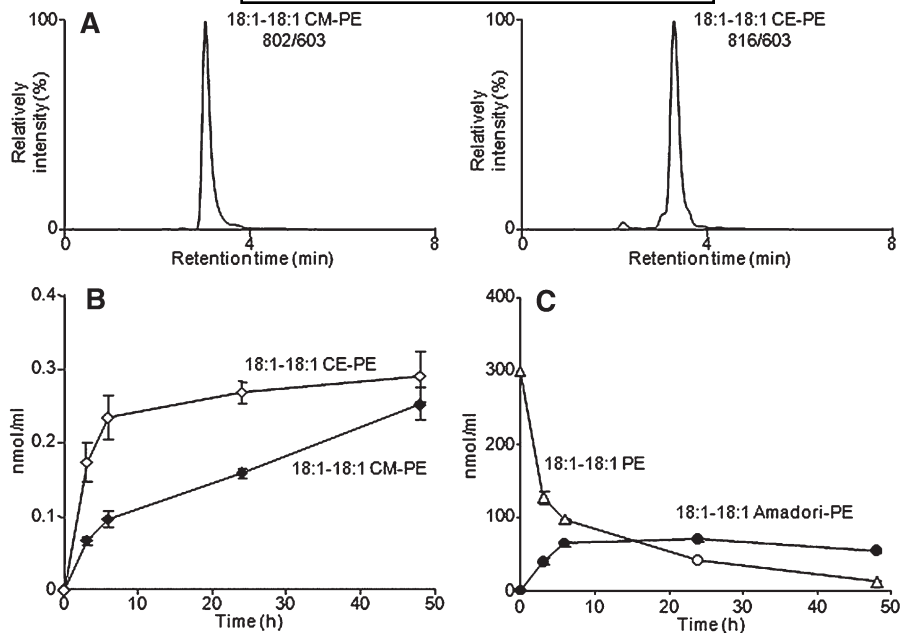


Fig. 3. In vitro lipid glycation and MRM detection of AGE-PE. 18:1-18:1 PE (9 μ mol) was incubated with glucose (15 mmol) in 30 ml of 0.1 M phosphate buffer-methanol (3:7, v/v, pH 7.4) at 60°C for 0–48 h. Typical MRM chromatograms of 18:1-18:1 CM-PE and 18:1-18:1 CE-PE when a lipid extract from the 48 h incubation mixture was analyzed by LC-MS/MS (A). Time course of changes in the amounts of 18:1-18:1 CM-PE and 18:1-18:1 CE-PE (B) and 18:1-18:1 Amadori-PE and 18:1-18:1 nonglycated PE (C). Values are means \pm SD ($n = 4$).

RESULTS

LC-MS/MS analysis of 18:1-18:1 CM-PE and 18:1-18:1 CE-PE

For the LC-MS/MS analysis of AGE-PE, we initially performed product ion scanning using synthesized 18:1-18:1 CM-PE and 18:1-18:1 CE-PE as reference compounds.

Product ion scanning indicated that protonated parent ions of 18:1-18:1 CM-PE (m/z 802 $[M+H]^+$) and 18:1-18:1 CE-PE (m/z 816 $[M+H]^+$) lost corresponding polar head groups ($H_2PO_4CH_2CH_2NHCH_2COOH$ and $H_2PO_4CH_2CH_2NHCHCH_3COOH$, respectively) upon collisional activation, yielding a characteristic fragment ion of diacylglycerol moiety (m/z 603) (Fig. 2A, B). The fragmentation was

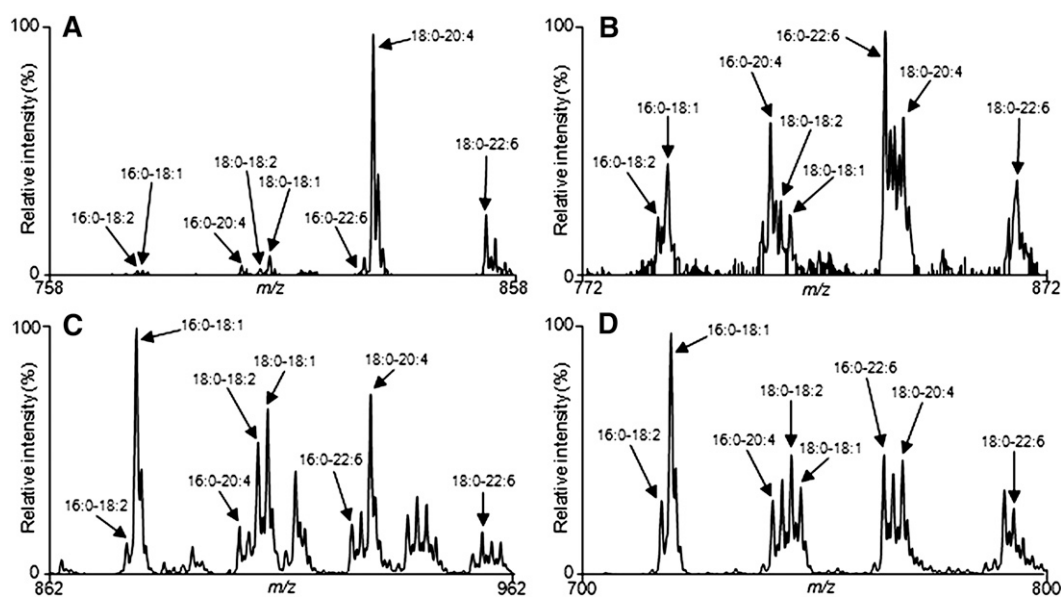


Fig. 4. NLS spectra of the erythrocyte extract from a healthy human subject. NLS of 199, 213, 303, and 141 Da show the specific detection of CM-PE (A), CE-PE (B), Amadori-PE (C), and nonglycated PE (D) species in erythrocytes, respectively. Total lipids were extracted from packed cells with 2-propanol and chloroform (17). The lipid extract was dissolved in 1 ml of chloroform-methanol (2:1, v/v), and a portion of the extract (5 μ l) was injected directly into MS/MS by an LC pump at the eluent (methanol) flow rate of 0.2 ml/min.

useful for MRM detection of AGE-PE in LC-MS/MS analysis. For instance, when 18:1-18:1 PE was incubated with glucose in vitro, 18:1-18:1 CM-PE and 18:1-18:1 CE-PE were clearly identified by LC-MS/MS with MRM (Fig. 3A).

The data indicated that CM-PE and CE-PE were actually produced as AGE products of PE (Fig. 3B, C).

Profiling of AGE-PE molecular species in blood samples

NLS of 199 Da and 213 Da yielded the parent ion of 18:1-18:1 CM-PE (m/z 802) and 18:1-18:1 CE-PE (m/z 816), respectively (Fig. 2C, D). We therefore used NLS of 199 Da and 213 Da for profiling the molecular species of CM-PE and CE-PE, respectively. When erythrocyte extract from a healthy subject was directly injected to MS/MS, a series of ion peaks corresponding to CM-PE and CE-PE molecular species (e.g., 16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6) were shown in the neutral loss spectra of 199 Da and 213 Da, respectively

(Fig. 4A, B). Amadori-PE and nonglycated native PE species were also detected by NLS of 303 Da ($H_2PO_4CH_2CH_2NHC_6H_{11}O_5$) and 141 Da ($H_2PO_4CH_2CH_2NH_2$), respectively (Fig. 4C, D). The neutral loss spectra of CM-PE, CE-PE, Amadori-PE, and native PE indicated that PE glycation proceeded toward the formation of AGE-PE in erythrocytes. The glycated molecular species of PE (CM-PE, CE-PE, and Amadori-PE molecular species) were also observed in NLS spectra of plasma samples; however, some AGE-PE species could not be clearly detected in plasma possibly due to their low amounts (supplementary Fig. 1).

Determination of AGE-PE in blood samples by LC-MS/MS with MRM

Based on the results of NLS, CM-PE and CE-PE molecular species (16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6) in erythrocytes and plasma were individually quantified by LC-MS/MS

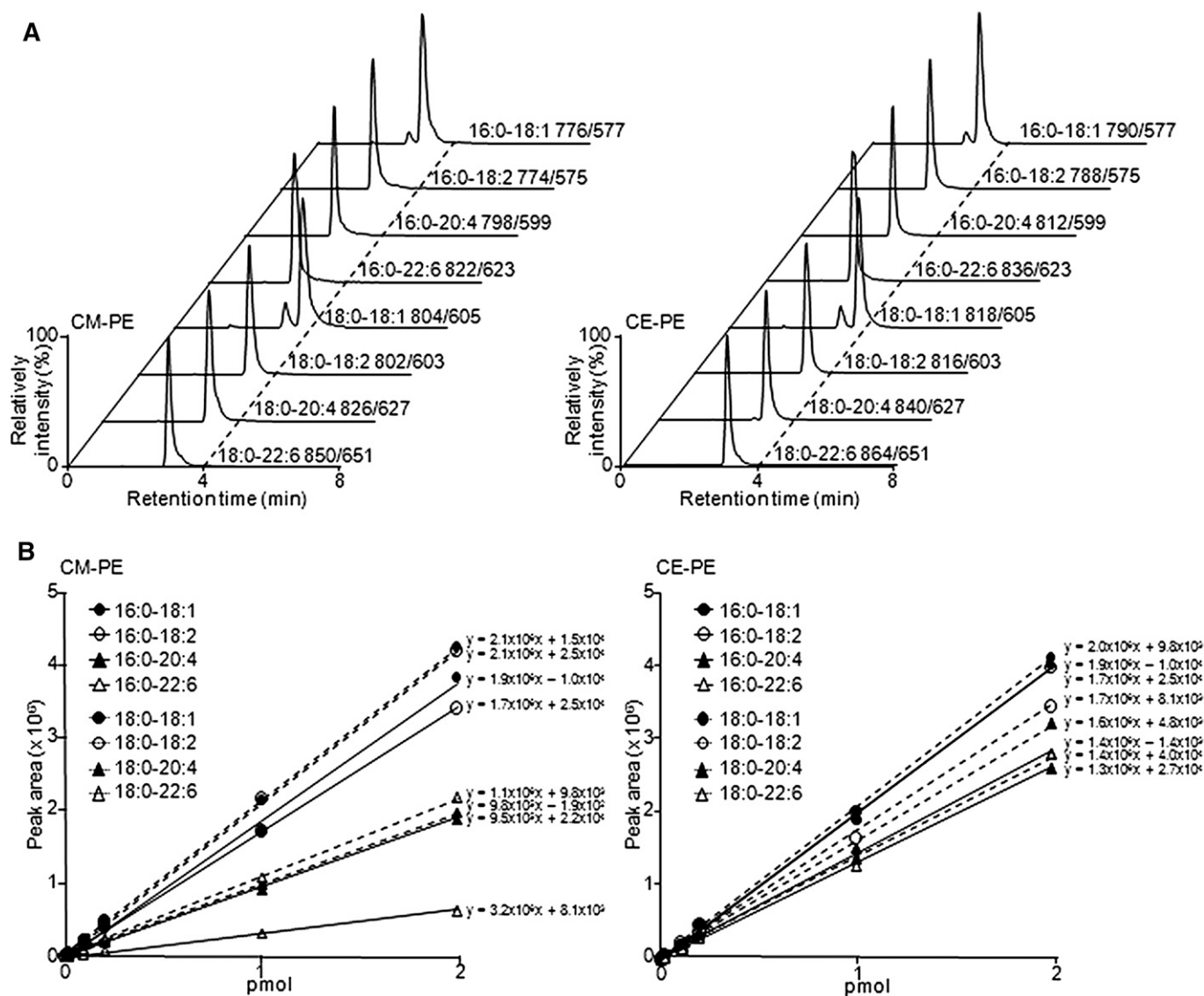


Fig. 5. Typical MRM chromatograms and calibration curves of CM-PE and CE-PE standards. A: Synthesized CM-PE or CE-PE (16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6; 1 pmol each) was analyzed by LC-MS/MS with MRM. B: The calibration curves were constructed with CM-PE and CE-PE standards of different concentrations (0.01–2 pmol/injection).

with MRM. Parameters were optimized to permit MRM detection and LC separation by using synthetic reference compounds (Fig. 5A). Under the optimized conditions, all calibration curves showed good linearity (0.998–0.999) (Fig. 5B), with detection limits of 5 fmol/injection at a signal-to-noise ratio of 3. The eight molecular species of both CM-PE and CE-PE were clearly detected in MRM chromatograms of erythrocytes (Fig. 6). Most of the AGE-PE molecular species were also shown in plasma MRM chromatograms (supplementary Fig. II). As shown in Tables 1 and 2, no significant differences were observed in CM-PE and CE-PE concentrations in erythrocytes and plasma between healthy subjects and diabetic patients. In contrast, Amadori-PE concentrations were significantly higher in diabetic erythrocytes and plasma (Tables 1 and 2).

DISCUSSION

For the occurrence of AGE of PE in vivo, there have been at least three conflicting reports (12–14). Requena et al. (12) and Pamplona et al. (13) detected hydrolysis product of CM-PE in the acid hydrolysates of human erythrocyte and mammalian mitochondrial phospholipids using GC-MS. These studies demonstrated the presence of AGE-PE in vivo; however, the GC-MS technique could not provide direct structural information (e.g., acyl chain composition) of AGE-PE. Breitling-Utzmann et al. (14) reported an LC-MS assay for AGE-PE. However, probably due to the insufficient sensitivity, they could not detect CM-PE and CE-PE in human erythrocytes.

A recently developed QTRAP MS/MS offers specific benefits (18) for biomolecular analysis including lipid molecules (7, 19, 20). The QTRAP MS/MS allows product ion scanning, NLS, and MRM, providing useful structural information of the analytes even in the presence of background contaminants in complex biological materials. In the present study, using synthesized CM-PE and CE-PE as reference compounds, we developed an LC-MS/MS

method to analyze AGE-PE with high selectivity and sensitivity at the molecular species level.

By using the QTRAP MS/MS, we found that protonated CM-PE and CE-PE tended to generate product ions of $[M+H-199]^+$ and $[M+H-213]^+$, respectively (Fig. 2). The neutral loss of the polar head group indicates that NLS (Fig. 2) and MRM (Fig. 3) are adaptable for the (LC-)MS/MS analysis of AGE-PE. The NLS technique enabled us to profile molecular species of CM-PE and CE-PE in human erythrocytes and plasma even without LC separation (Fig. 4, supplementary Fig. I). These results provide direct information on the molecular species of AGE-PE in erythrocytes and plasma and indicate that PE glycation proceeds toward the formation of AGE-PE in vivo. However, compared with native PE molecular species composition, 18:0-20:4 and 18:0-22:6 species were higher in erythrocyte CM-PE composition (Fig. 4, Table 1). It suggests that 18:0-20:4 and 18:0-22:6 PE are susceptible to carboxymethylation or these CM-PE molecular species are more stable than the others in erythrocytes, but the possibilities require investigation. In this study, we analyzed glycation products of diacyl PE species; however, alkenyl-acyl PE (plasmalogen) species are also in biological specimens. It appears likely that glycated alkenyl-acyl PE species are also generated in vivo. However, due to the limited source of pure alkenyl-acyl PE species, it is difficult at present to prepare plasmenyl AGE-PE molecular species as authentic standards for LC-MS/MS analysis. Thus, we could not evaluate the plasmenyl AGE-PE species in this study.

MRM experiments can provide accurate quantitation of lipid molecules, as reviewed by Sullards (21). In the present study, LC-MS/MS with MRM was highly useful for the measurement of AGE-PE in blood samples. Based on the results of NLS, we focused on eight molecular species (16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6) of both CM-PE and CE-PE in erythrocytes and plasma and determined individual

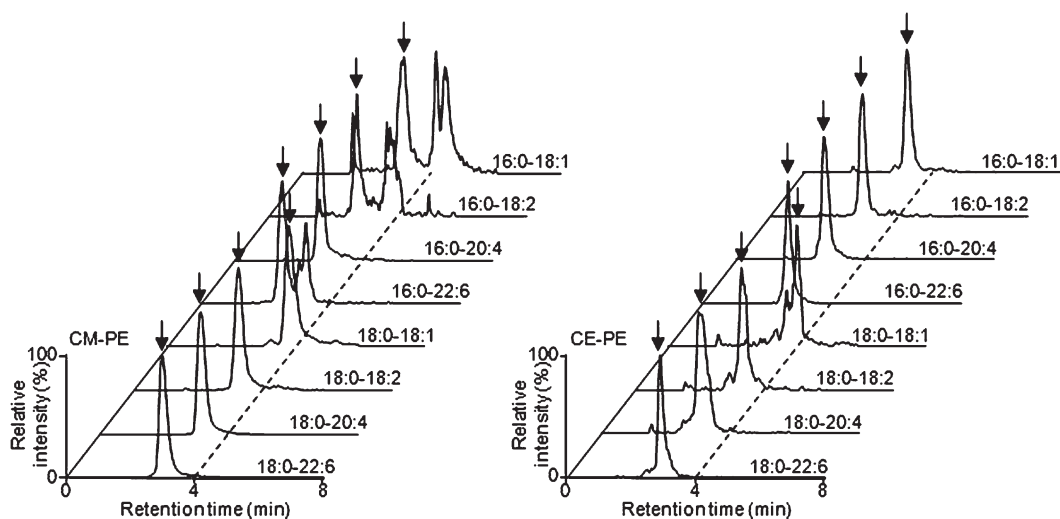


Fig. 6. Typical MRM chromatograms of CM-PE and CE-PE molecular species in human erythrocytes. An erythrocyte lipid extract (2 μ l) of a healthy human subject was subjected to LC-MS/MS with MRM.

TABLE 1. Lipid glycation products in erythrocytes of healthy subjects and diabetic patients

Molecular Species	Packed Cells						Packed Cells	
	CM-PE		CE-PE		Amadori-PE		Native PE	
	Healthy	Diabetic	Healthy	Diabetic	Healthy	Diabetic	Healthy	Diabetic
			<i>pmol/ml</i>				<i>nmol/ml</i>	
16:0-18:1	5 ± 6	2 ± 1	15 ± 6	11 ± 4	150 ± 22	621 ± 217 ^a	54 ± 9	60 ± 7
16:0-18:2	1 ± 0	0 ± 0	6 ± 2	5 ± 1	9 ± 1	33 ± 11 ^a	29 ± 5	30 ± 5
16:0-20:4	5 ± 2	7 ± 5	14 ± 5	12 ± 5	124 ± 18	462 ± 168 ^a	209 ± 32	197 ± 29
16:0-22:6	9 ± 7	11 ± 6	10 ± 4	10 ± 4	55 ± 14	198 ± 58 ^a	278 ± 63	332 ± 86
18:0-18:1	15 ± 5	18 ± 12	5 ± 2	3 ± 1	107 ± 18	428 ± 165 ^a	20 ± 2	21 ± 4
18:0-18:2	9 ± 7	17 ± 16	7 ± 2	4 ± 1	72 ± 16	251 ± 99 ^a	29 ± 4	29 ± 5
18:0-20:4	423 ± 60	567 ± 452	15 ± 3	12 ± 3	179 ± 34	714 ± 311 ^a	123 ± 15	134 ± 21
18:0-22:6	60 ± 16	82 ± 60	10 ± 2	11 ± 3	17 ± 5	86 ± 30 ^a	53 ± 6	56 ± 6
Total	528 ± 83 ^b (0.67 ± 0.09) ^c	705 ± 533 (0.80 ± 0.53)	82 ± 24 (0.10 ± 0.04)	68 ± 16 (0.08 ± 0.02)	712 ± 52 (0.91 ± 0.11)	2,793 ± 989 ^a (3.38 ± 1.59) ^a	794 ± 106	859 ± 110

Values are means ± SD (n = 8 for healthy subjects and n = 10 for diabetic patients).

^a P < 0.01 compared with healthy subjects.

^b Concentrations of lipid glycation products are presented as sum of molecular species of 16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6.

^c Number in parenthesis represents as mmol/mol of total CM-PE, CE-PE, or Amadori-PE species against total native PE species.

concentrations using MRM. For LC separation, we investigated LC conditions and adopted an ODS column for separation of AGE-PE species. A silica column under hydrophilic interaction chromatography mode was used for separation of both Amadori-PE and nonglycated PE. These LC conditions could reduce background noise and improve resolutions of the analytes. Under the present conditions, the detection limits of AGE-PE by MRM were around 5 fmol/injection, which were relatively sensitive compared with that of LC analysis of phospholipid derivatives, such as phospholipid hydroperoxides (22, 23) and platelet-activating factor-like phospholipids (24) (detection limits above picomole levels). Synthetic CM-PE and CE-PE standards permitted the quantitation of predominant AGE-PE molecular species in human erythrocytes and plasma (Figs. 5 and 6, supplementary Fig. II). In this study, however, interfering peaks in MRM chromatograms made it difficult to determine minor AGE-PE molecular species (e.g., 18:0-18:1 CE-PE) in plasma (supplementary Fig. II, Table 2).

According to the protein glycation mechanism, the generation of CM derivatives requires an oxidation step. The reaction proceeds via the oxidative degradation of Amadori products (25) and/or the reaction of amines with glyoxal, a product of glucose autooxidation as well as lipid peroxidation (26, 27). On the other hand, CE derivatives (28) are reaction products of the C-3 unit methylglyoxal, which is a product formed by reverse aldol reaction of 3-deoxyosones, enzymatic synthesis from dihydroxyacetone phosphate, and nonenzymatic dephosphorylation of glyceraldehyde phosphate or dihydroxyacetone phosphate. Considering this literature (25–28), the plausible formation mechanism of CM-PE and CE-PE is depicted in Fig. 1B. The different formation mechanisms between CM-PE and CE-PE may explain why the CM-PE level is higher than that of CE-PE in vivo (Tables 1 and 2). In addition, CM-PE and CE-PE might be used as biomarkers for oxidative stress and carbonyl stress, respectively.

In the present study, among the glycation products of PE, only Amadori-PE was significantly elevated in erythrocytes

TABLE 2. Lipid glycation products in plasma of healthy subjects and diabetic patients

Molecular species	CM-PE		CE-PE		Amadori-PE		Native PE	
	Healthy	Diabetic	Healthy	Diabetic	Healthy	Diabetic ^a	Healthy	Diabetic
			<i>pmol/ml</i>				<i>nmol/ml</i>	
16:0-18:1	0.3 ± 0.3	0.2 ± 0.1	<0.1	0.1 ± 0.1	8 ± 6	27 ± 13	0.3 ± 0.2	0.8 ± 0.4 ^a
16:0-18:2	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	4 ± 1	9 ± 3	1.3 ± 0.4	2.5 ± 1.0 ^a
16:0-20:4	0.6 ± 0.3	0.6 ± 0.3	0.4 ± 0.3	0.6 ± 0.3	39 ± 22	159 ± 75	3.5 ± 1.0	6.0 ± 2.1 ^a
16:0-22:6	2.4 ± 2.0	2.5 ± 1.0	0.9 ± 0.5	1.9 ± 0.7	15 ± 7	95 ± 61	25.1 ± 7.1	62.7 ± 17.3 ^a
18:0-18:1	0.5 ± 0.2	0.3 ± 0.2	<0.1	<0.1	7 ± 3	25 ± 10	1.0 ± 0.4	1.3 ± 0.5
18:0-18:2	0.9 ± 0.5	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	23 ± 8	72 ± 39	2.9 ± 1.0	4.4 ± 1.9
18:0-20:4	2.9 ± 1.9	1.8 ± 1.1	0.4 ± 0.2	0.5 ± 0.2	64 ± 23	324 ± 183	10.9 ± 3.8	14.3 ± 5.1
18:0-22:6	0.4 ± 0.2	0.9 ± 0.8	<0.1	0.4 ± 0.3	6 ± 2	46 ± 28	10.5 ± 3.7	26.9 ± 8.9 ^a
Total	7.7 ± 3.5 ^b (0.15 ± 0.07) ^c	6.6 ± 3.1 (0.06 ± 0.02)	2.5 ± 1.1 (0.05 ± 0.03)	4.2 ± 1.5 (0.04 ± 0.01)	165 ± 66 (2.97 ± 0.88)	757 ± 377 (6.30 ± 1.91)	55.4 ± 15.6	119 ± 33.9 ^a

Values are means ± SD (n = 8 for healthy subjects and n = 10 for diabetic patients).

^a P < 0.01 compared with healthy subjects.

^b Concentrations of lipid glycation products are presented as sum of molecular species of 16:0-18:1, 16:0-18:2, 16:0-20:4, 16:0-22:6, 18:0-18:1, 18:0-18:2, 18:0-20:4, and 18:0-22:6.

^c Number in parenthesis represents as mmol/mol of total CM-PE, CE-PE, or Amadori-PE species against total native PE species.

and plasma of diabetic subjects (Tables 1 and 2). The data suggest that hyperglycemia in diabetic patients does not affect AGE-PE concentrations in erythrocytes and plasma, whereas the Amadori-PE concentration was markedly increased under hyperglycemic conditions. Similar to the present results, Requena et al. (12) reported that no differences were observed in erythrocyte CM-PE levels between healthy and diabetic subjects. The reason given was that the diabetic patients participating in that study were free of complications (12). Thus, AGE-PE might have accumulated more in diabetic patients with severe complications, because oxidative stress and carbonyl stress under hyperglycemic conditions are considered to be involved in the pathogenesis of diabetic complications. In this study, like AGE-PE, no significant differences were observed in plasma concentrations of carboxymethyllysine (one of the well-known protein AGEs) between healthy subjects and diabetic patients (data not shown). Therefore, in a future study, it should be necessary to further elucidate the involvement of AGE lipids and AGE proteins in the pathogenesis of diabetic complications by analyzing their levels between diabetic patients with and without complications. On the other hand, as mentioned above, we found that Amadori-PE, but not carboxymethyllysine, was higher in diabetic blood. The result suggests that Amadori-PE is more prone to be accumulated compared with carboxymethyllysine, even in early stages of diabetes. This may be related to the fact that Amadori-PE and carboxymethyllysine are early and advanced glycation products, respectively (29, 30). To put it another way, Amadori-PE may be used as a potentially sensitive marker for reflecting hyperglycemic conditions in the early stage of diabetes (31–33).

In summary, we developed the LC-MS/MS assay for CM-PE and CE-PE and provided direct information on the molecular species of AGE-PE in human erythrocytes and plasma. The LC-MS/MS technique with MRM will be a powerful tool for understanding the pathophysiological consequence of in vivo lipid glycation.

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