Ion-trap tandem mass spectrometric analysis of Amadori-glycated phosphatidylethanolamine in human plasma with or without diabetes

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Abstract **Peroxidized phospholipid-mediated cytotoxicity is involved in the pathophysiology of diseases [i.e., an abnormal increase of phosphatidylcholine hydroperoxide (PCOOH) in plasma of type 2 diabetic patients]. The PCOOH accumulation may relate to Amadori-glycated phosphatidylethanolamine (Amadori-PE; deoxy-D-fructosyl phosphatidylethanolamine), because Amadori-PE causes oxidative stress. However, the occurrence of lipid glycation products, including Amadori-PE, in vivo is still unclear. Consequently, we developed an analysis method of Amadori-PE using a quadrupole/linear ion-trap mass spectrometer, the Applied Biosystems QTRAP. In positive ion mode, collision-induced dissociation of Amadori-PE produced a well-characterized diglyceride ion ($[M+H-303]^+$) permitting neutral loss scanning and multiple reaction monitoring (MRM). When lipid extract from diabetic plasma was infused directly into the QTRAP, Amadori-PE molecular species could be screened out by neutral loss scanning. Interfacing liquid chromatography with QTRAP mass spectrometry enabled the separation and determination of predominant plasma Amadori-PE species with sensitivity of ~0.1 pmol/injection in MRM. The plasma Amadori-PE level was 0.08 mol% of total PE in healthy subjects and 0.15–0.29 mol% in diabetic patients. Furthermore, plasma Amadori-PE levels were positively correlated with PCOOH (a maker for oxidative stress). These results show the involvement between lipid glycation and lipid peroxidation in diabetes pathogenesis.**—Nakagawa, K., J-H. Oak, O. Higuchi, T. Tsuzuki, S. Oikawa, H. Otani, M. Mune, H. Cai, and T. Miyazawa. **Ion-trap tandem mass spectrometric analysis of Amadori-glycated phosphatidylethanolamine in human plasma with or without diabetes.** ***J. Lipid Res.*** **2005.** 46: **2514–2524.**

Supplementary key words glycation • QTRAP mass spectrometer • lipid peroxidation

Manuscript received 15 July 2005 and in revised form 9 August 2005.

Published, JLR Papers in Press, September 8, 2005.
DOI 10.1194/jlr.D500025-JLR200

Oxidative stress on lipids plays a role in the pathophysiology of atherogenesis, diabetes, aging, and other conditions (1). To determine lipid hydroperoxides as a primary oxidation product, we established the chemiluminescence detection-liquid chromatography method (2). Using this method, it was confirmed that plasma phosphatidylcholine hydroperoxide (PCOOH) increases in hyperlipidemic patients (3) and in type 2 diabetic patients (4). Hence, we hypothesized that plasma PCOOH formation is closely involved in the pathophysiology of these diseases.

To understand why PCOOH increases in diabetic plasma, we have studied "lipid glycation" (5). Lipid glycation refers to the reaction between aminophospholipids such as phosphatidylethanolamine (PE) and glucose. The reaction involves a Schiff base formation and its rearrangement to PE-linked Amadori product (Amadori-PE) (**Fig. 1**) (5, 6). Therefore, it is likely that PE is exposed to glycation under hyperglycemic conditions, yielding Amadori-PE in vivo. Amadori-PE causes oxidative stress (i.e., PCOOH formation) (7), leading to a disorder of cellular integrity (i.e., angiogenesis stimulation) (8). Consequently, Amadori-PE and PCOOH may play a role in the development of diabetes.

Despite the potential significance of Amadori-PE in pathological signaling, its characteristics and a quantitative method in vivo have not been established. Accordingly, we developed a liquid chromatography method using an ultraviolet-labeling reagent (9). This allowed us to

Abbreviations: AGE, advanced glycation end product; Amadori-PE, Amadori-glycated phosphatidylethanolamine; Hb_{A1c}, hemoglobin A1c; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PEOOH phosphatidylethanolamine hydroperoxide; PS, phosphatidylserine; QqLIT, hybrid quadrupole/linear ion trap.

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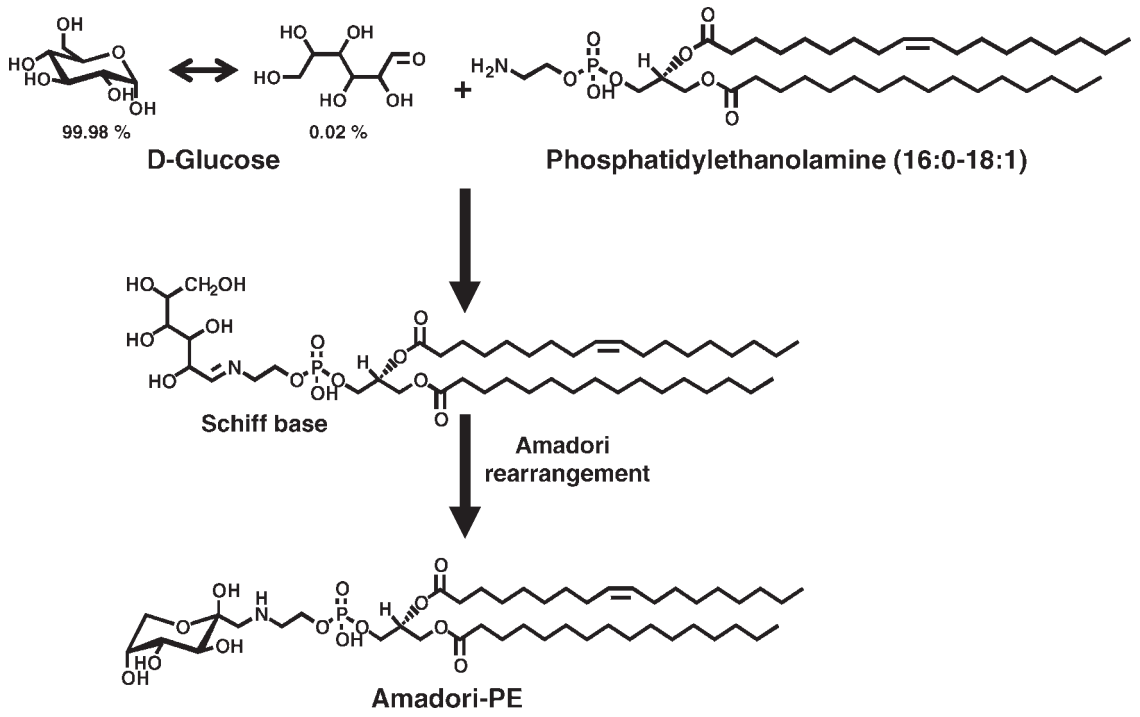

Fig. 1. Scheme for the glycation of phosphatidylethanolamine (PE). Glucose reacts with the amino group of PE to form an unstable Schiff base, which undergoes an Amadori rearrangement to yield the stable PE-linked Amadori product (Amadori-PE; deoxy-D-fructosyl phosphatidylethanolamine).

show the occurrence of Amadori-PE in foodstuffs. However, we were unable to detect Amadori-PE in biological samples, because of insufficient sensitivity. On the other hand, there are a few reports of Amadori-PE analysis using liquid chromatography with online mass spectrometry (10, 11). These reports suggested the presence or near absence of Amadori-PE in blood plasma, red blood cells, and atherosclerotic plaques from human. During LC-MS analysis, Amadori-PE can only be identified by its molecular ion. In addition, the Amadori-PE peak appeared concomitant with background contaminants from biological samples. Consequently, to ascertain Amadori-PE generation in vivo, another efficient method is required.

A recently developed hybrid quadrupole/linear ion trap (QqLIT) spectrometer, QTRAP, offers special benefit as a liquid chromatography-tandem mass spectrometry (LC-MS/MS) detector for biomolecular analysis (12–14). With the advent of QTRAP, both triple quadrupole and ion-trap scans can be performed together in a single stage. The product ion scan, neutral loss scan, and multiple reaction monitoring (MRM) can provide useful structural information about the analyte, even in the presence of major background contaminants from complex biological matrices. Recently, the analysis of lipids (i.e., sphingolipids and phospholipids) using QTRAP has been reported (12).

Based on this knowledge, we developed a QTRAP LC-MS/MS method for Amadori-PE in plasma of human with or without diabetes. Using the method, we confirmed that Amadori-PE is present at higher levels in plasma of diabetic patients compared with healthy subjects, and we discuss the possible peroxidative role of Amadori-PE in vivo.

MATERIALS AND METHODS

Materials

1-Hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine (16:0-18:1 PE), 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphoethanolamine (16:0-20:4 PE), 1-hexadecanoyl-2-docosahex-4′,7′,10′,13′,16′,19′-enoyl-*sn*-glycero-3-phosphoethanolamine (16:0-22:6 PE), and 1,2-di-octadec-9′-enoyl-*sn*-glycero-3-phosphoethanolamine (18:1-18:1 PE) were purchased from Funakoshi (Tokyo, Japan). (The molecular species of Amadori-PE and PE are indicated by the total number of carbons in the acyl chains at the *sn*-1,2-positions and the degree of unsaturation.) Using these PE species, Amadori-PE standards were synthesized as described previously (7). Standard solutions were prepared by dissolving the Amadori-PE or PE in a mixture of methanol-water (99:1, v/v; containing 5 mM ammonium acetate) to reach a concentration of 10 nmol/ml. All other reagents were of the highest grade available.

Instruments

The QTRAP LC-MS/MS system consisted of an Agilent 1100 series liquid chromatograph (Palo Alto, CA), including a vacuum degasser, a quaternary pump, and an autosampler, coupled with an Applied Biosystems 4000 QTRAP tandem mass spectrometer (Foster City, CA) equipped with a turbo ion spray source. This instrument is based on a triple quadrupole ion path in which the final quadrupole can be used as a QqLIT mass spectrometer.

Blood samples and lipid extraction

Human blood samples were collected from five healthy volunteers [mean age, 28 ± 11 years; hemoglobin A1c (Hb_{A1c}), 4.5 ± 0.1%], nine type 2 diabetic patients (mean age, 68 ± 31 years; Hb_{A1c}, 6.9 ± 2.1%), five diabetic patients on chronic hemodialysis (mean age, 76 ± 7 years; Hb_{A1c}, 7.6 ± 3.0%), and five nondia-

betic patients on chronic hemodialysis (mean age, 65 ± 8 years; Hb_{A1c}, 4.7 ± 0.8%) after informed consent had been obtained. For diabetic patients, their blood sugar was under control (insulin or diet) at the time of blood draw. Plasma was prepared from heparinized blood by centrifugation at 1,000 *g* for 15 min at 4°C.

As described by Folch, Lees, and Sloane-Stanley (15), total lipids were extracted from plasma (1 ml) using 4 ml of chloroform-methanol (2:1, v/v). The extract was partitioned by centrifugation at 1,000 *g* for 10 min at 4°C into two phases: the chloroform layer (lower organic phase) and the methanol-water layer (upper phase). The lower chloroform layer (lipid fraction) was collected. The remaining aqueous methanol layer containing semisolid interface was mixed with 1 ml of 0.15 M NaCl and 2 ml of chloroform-methanol (2:1) and subjected to centrifugation at 1,000 *g* for 10 min at 4°C. This reextraction was repeated twice. After evaporation of the combined chloroform layer (plasma total lipid fraction), the dried extract was redissolved in 100 μl of a mixture of methanol-water (99:1, v/v; containing 5 mM ammonium acetate).

Qualitative MS/MS analysis of plasma Amadori-PE

Neutral loss scanning was used for the qualitative analysis of Amadori-PE. Ten microliters of plasma extract was infused directly into the QTRAP by a flow syringe pump at 0.01 ml/min. The positive ionization mode was adopted. MS parameters including collision energy and spraying conditions were optimized with Amadori-PE standards. The QTRAP was programmed to scan the protonated molecules that had a neutral loss of 303 Da after collision-activated dissociation at the collision cell (Q2; collision gas, N_2; 4 pounds per square inch; collision energy, 30 eV) over the *m/z* range from 100 to 1,200. Turbo gas temperature was kept at 400°C, and the spray voltage was 5,500 V. Nitrogen values for the turbo, nebulizer, and curtain gases were set at 70, 50, and 20 pounds per square inch, respectively.

Quantitative LC-MS/MS analysis of plasma Amadori-PE

Plasma extract (10 μl) was separated on a TSK gel ODS 100s (2.0 × 150 mm; Tosho, Tokyo, Japan) fitted with a guard column (Inertsil ODS-3; 3.0 mm × 10 mm; GL Science, Tokyo, Japan). The column was eluted with a mixture of methanol-water (99.5: 0.5, v/v; containing 5 mM ammonium acetate) at a flow rate of 0.3 ml/min, and the column temperature was maintained at 30°C. At post column, predominant plasma Amadori-PE species were individually detected by the QTRAP using MRM for the transition of parent ion to product ion. The collision energy for transition was 30 eV. The dwell time for transition was 100 ms. Other MS conditions were the same as those for the qualitative method.

To obtain quantitative results, before the extraction process, plasma (1 ml) was spiked with different amounts of Amadori-PE standards (16:0-18:1, 16:0-20:4, and 16:0-22:6; each 0–100 pmol). To obtain the recovery rate of Amadori-PE, a constant amount (10 pmol) of nonnaturally occurring 18:1-18:1 Amadori-PE (internal standard) was also added to plasma. After that, Amadori-PE was extracted and detected by LC-MS/MS with MRM, according to the procedures described above. Calibration curves of Amadori-PE standards were constructed using the peak area ratios of the internal standard. Amadori-PE concentrations in unknown plasma samples were then calculated from their peak area ratios versus calibration curves. Plasma native PE molecular species (16:0-18:1, 16:0-20:4, and16:0-22:6) were determined by LC-MS/MS with MRM, and the mol% of Amadori-PE to PE was calculated.

Other assays

PCOOH and phosphatidylethanolamine hydroperoxide (PEOOH) in plasma were simultaneously determined by the chemiluminescence detection-LC method, as described previously (2). Fasting blood glucose, Hb_{A1c}, and lipids (cholesterol, triglyceride, and phospholipid) in plasma were determined enzymatically with an autoanalyzer [GAO2U (A&T Co., Tokyo, Japan); HLC-723GHbIIIs (Tosoh Co., Tokyo, Japan); and H7350 Automatic Analyzer (Hitachi Co., Tokyo, Japan), respectively].

Statistics

The data are expressed as means ± SD and analyzed using Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Amadori-PE molecular species

Amadori-PE is composed of a glycerol backbone with two fatty acids and a glycated moiety ($-HPO_4CH_2CH_2NHC_6H_{11}O_5$) esterified on the *sn*-1,2 and *sn*-3 positions, respectively (Fig. 1). Here, the glycated moiety on the *sn*-3 position is referred to as the polar head group.

MS fragmentation of Amadori-PE

The MS analysis of Amadori-PE was primarily investigated using the QTRAP with flow injection. A protonated ion of Amadori-PE standards (i.e., 16:0-18:1, 16:0-20:4, 16:0-22:6, and 18:1-18:1 Amadori-PE) was constantly observed in the Q1 mass spectrum (data not shown). Next, to understand the fragmentation characteristics of Amadori-PE, product ion scanning was performed using Q3 in QqLIT mode (**Fig. 2A**). For instance, 18:1-18:1 Amadori-PE showed a $[M+H]^+$ ion at *m/z* 906. Upon collisional activation, the $[M+H]^+$ loses a polar head group [303 Da ($H_2PO_4CH_2CH_2NHC_6H_{11}O_5$)], yielding a characteristic diglyceride ion (*m/z* 603 $[M+H-303]^+$). The same fragmentation pattern was observed for other Amadori-PE standards, indicating that the $[M+H-303]^+$ is specifically diagnostic for Amadori-PE. Thus, by exploiting the loss of the polar head group (303 Da), Amadori-PE can be spectroscopically isolated. As anticipated, only a molecular ion could be detectable in the neutral loss spectrum of 18:1-18:1 Amadori-PE (Fig. 2B). This result opens the possibility that Amadori-PE can be sought out from biological samples with high confidence using a neutral loss scan of 303 Da. Additionally, nonglycated PE can be detected by scanning for a neutral loss of 141 Da ($H_2PO_4CH_2CH_2NH_2$) (Fig. 2C).

Specific detection of plasma Amadori-PE by neutral loss scanning

To ascertain the occurrence of Amadori-PE in vivo, plasma samples from diabetic patients were subjected to QTRAP analysis with flow injection. Notable peaks corresponding to Amadori-PE molecular species were indeed observed in the neutral loss (303 Da) spectrum (**Fig. 3A**) but not in the Q1 mass spectrum (data not shown). The fragmentation pattern of endogenous Amadori-PE species was identical to that of standards (**Fig. 4**). Fatty acid composition and distribution could be assigned based on the intensity ratio of *sn*-2/*sn*-1 carboxylate ions by negative MS/MS or MS/MS/MS experiments (data not shown) (12). It was found that the predominant molecular species of Amadori-PE in diabetic plasma were 16:0-18:1 (*m/z* 880), 16:0-

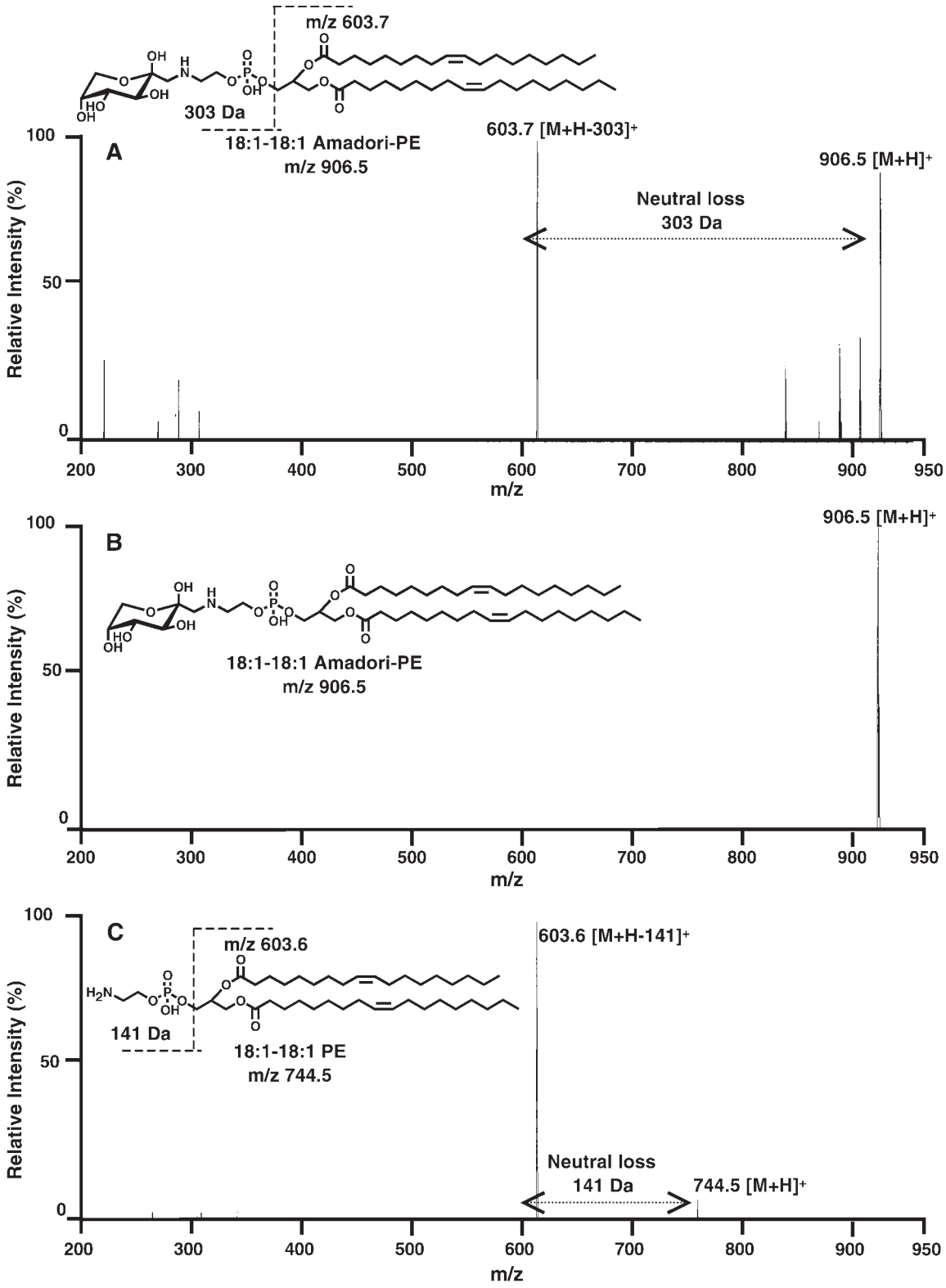

Fig. 2. Specific detection of Amadori-PE and PE standards using the QTRAP mass spectrometer. A: Product ion spectrum of the $[M+H]^+$ ion at *m/z* 906 of 18:1-18:1 Amadori-PE. B: Neutral loss scan for 303 Da showing specific detection of 18:1-18:1 Amadori-PE. C: Product ion spectrum of nonglycated PE (18:1-18:1 PE, *m/z* 744 $[M+H]^+$). The standard solution of Amadori-PE or PE (each 10 nmol/ml) was infused directly into the QTRAP using a syringe pump at a flow rate of 0.01 ml/min.

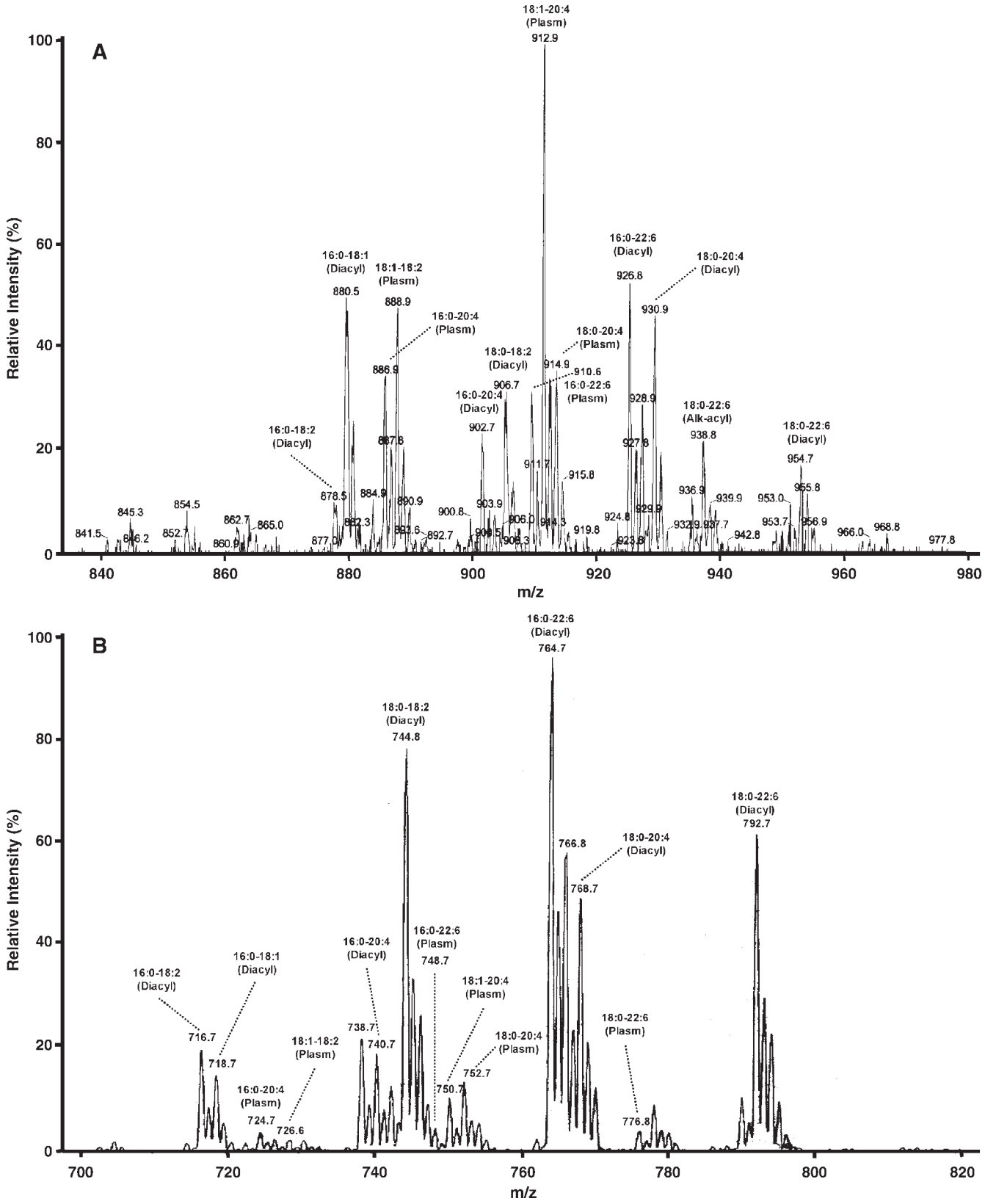

Fig. 3. Neutral loss scan spectra of the plasma extract of a diabetic patient. A: Neutral loss of 303 Da showing the specific detection of plasma Amadori-PE species. B: Neutral loss of 141 Da for nonglycated PE detection. Total lipids were extracted from plasma of a diabetic patient without chronic hemodialysis [female, 72 years old; fasting blood glucose, 185 mg/dl; hemoglobin A1c (Hb_{A1c}), 7.2] by the method of Folch, Lees, and Sloane-Stanley (15). The lipid extract was redissolved in 100 μl of methanol-water (99:1, v/v; containing 5 mM ammonium acetate) and infused directly into the QTRAP using a syringe pump at a flow rate of 0.01 ml/min.

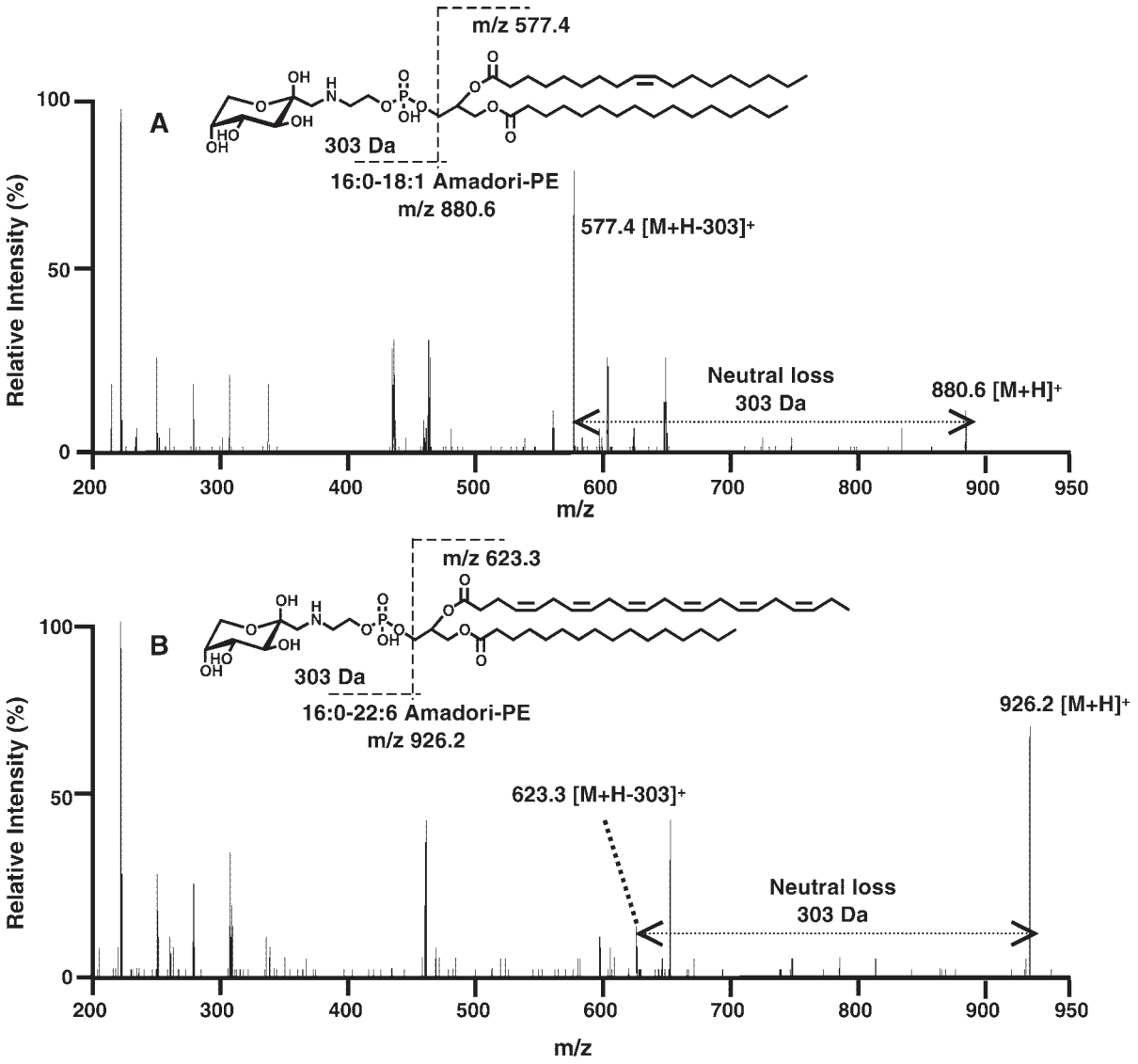

Fig. 4. Fragmentation patterns of the ion peaks of 16:0-18:1 Amadori-PE (*m/z* 880) and 16:0-22:6 Amadori-PE (*m/z* 926) detected in the neutral loss (303 Da) spectrum of Fig. 3A.

18:2 (*m/z* 878), 16:0-20:4 (*m/z* 902), 16:0-22:6 (*m/z* 926), 18:0-18:2 (*m/z* 906), 18:0-20:4 (*m/z* 930), and 18:0-22:6 (*m/z* 954) as diacyl species and 16:0-20:4 (*m/z* 886), 16:0-22:6 (*m/z* 910), 18:0-20:4 (*m/z* 914), 18:0-22:6 (*m/z* 938), 18:1-18:2 (*m/z* 888), and 18:1-20:4 (*m/z* 912) as alkenyl-acyl species (plasmalogen). The neutral loss spectra of Amadori-PE and nonglycated PE indicated that the molecular species of PE were randomly glycated (Fig. 3). These results provided the first direct structural evidence for the existence of Amadori-PE in vivo.

Quantitative analysis of Amadori-PE by LC-MS/MS with MRM

MRM experiments addressed the issues regarding the accurate quantitation of lipid molecules, as reviewed by Sullards (16). Here, three predominant Amadori-PE species (16:0-18:1, 16:0-20:4, and 16:0-22:6) in plasma of human with or without diabetes were individually quantified by LC-MS/MS with MRM. Initially, parameters were optimized to permit MRM detection and LC separation (**Fig. 5A**) of Amadori-PE standards (16:0-18:1, 16:0-20:4, and 16:0-22:6). Then, calibration curves were established by spiking different amounts of Amadori-PE standards to sample plasma. All calibration curves showed good linearity (0.995–0.999), and detection limits were ~0.1 pmol/injection at a signal-to-noise ratio of 3 (**Figs.** 5A and **6A**). In the same way, parameters for LC-MS/MS with MRM could be optimized to detect nonglycated PE standards (Figs. 5A and 6B). Once these conditions were fully optimized, in MRM chromatograms of plasma of diabetic patients, native PE species (16:0-18:1, 16:0-20:4, and 16:0-22:6) as well as their Amadori products appeared as clear peaks (Fig. 5B). It was found that the plasma 16:0-18:1 Amadori-PE concentration was ~3 pmol/ml in healthy subjects and 6–11 pmol/ml in diabetic patients. When calculating the 16:0-18:1 Amadori-PE/16:0-18:1 PE mol%, we found that amounts of Ama-

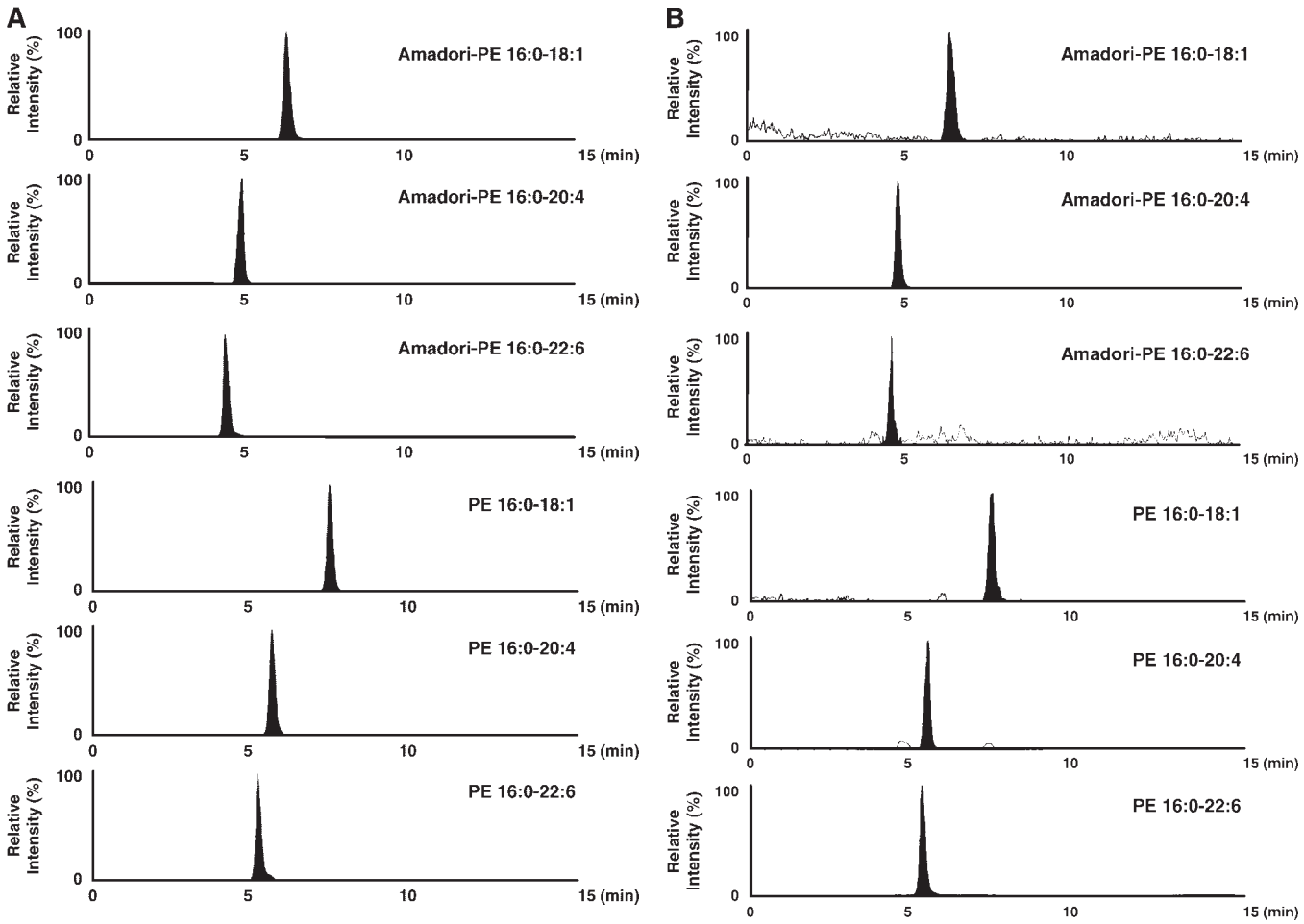

Fig. 5. Typical multiple reaction monitoring (MRM) chromatograms of Amadori-PE and PE species. A: Standard Amadori-PE or PE (16:0-18:1, 16:0-20:4, and 16:0-22:6; each 10 pmol) was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with MRM. B: Plasma lipid extract (10 μl) of a diabetic patient (female, 72 years old; fasting blood glucose, 185 mg/dl; Hb_{A1c}, 7.2) was subjected to LC-MS/MS with MRM.

dori-PE in plasma of diabetic patients (0.15 mol%), diabetic patients on chronic hemodialysis (0.29 mol%), and nondiabetic patients on chronic hemodialysis (0.13 mol%) were higher than that of the control group (0.08 mol%) (**Fig. 7A**). Similarly, average glycation rates of 16:0-20:4 Amadori-PE/16:0-20:4 PE and of 16:0-22:6 Amadori-PE/16:0-22:6 PE in diabetic plasma were ~0.13–0.30 mol%, which were higher than that of healthy controls (0.05–0.10 mol%) (data not shown). High reproducibility for plasma Amadori-PE/PE mol% (coefficient of variability $< 7\%$) was confirmed and was not altered by storage of plasma at −80°C for 1 week. It is notable that plasma Amadori-PE levels were positively correlated with plasma PCOOH (an oxidative stress marker) (Fig. 7B, C).

DISCUSSION

Proteins react nonenzymatically with glucose, leading to the production of unstable Schiff bases and ultimately Amadori products. These early glycation products are further transformed into a wide spectrum of compounds termed advanced glycation end products (AGEs) (17). Numerous studies have described the formation and accumulation of Amadori products, such as glycated hemoglobin and serum albumin, and AGEs, such as carboxymethyllysine and carboxyethyllysine, in blood and a range of tissues (18–21). This protein glycation progresses during normal aging and at an extremely accelerated rate in patients with diabetes mellitus (22). These changes have been implicated in the development of diabetic vascular complications and thereby contribute to the disabilities and high mortality rate found in diabetic patients (23, 24).

Although glycation of proteins has been investigated thoroughly, little attention has been paid to lipid glycation. Because of the presence of a free amino group, it seemed logical that, like proteins, aminophospholipids can be modified by glycation (6). In vivo, lipid glycation is likely to induce changes in the biosynthesis and turnover of membrane phospholipids, the physical properties of membranes, the activity of membrane-bound enzymes, and the susceptibility to oxidative stress. These changes may contribute to the pathology of aging and chronic disease, such as diabetes and atherosclerosis. The increasing importance of lipid glycation in human nutrition and physiology has shown the need for analytical means to detect lipid glycation com-

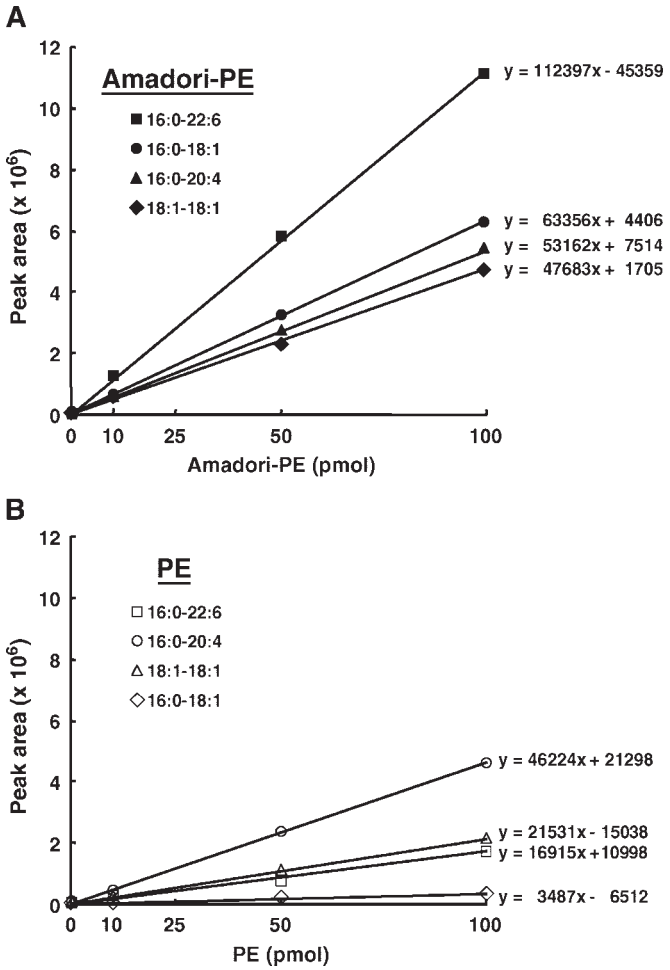

Fig. 6. Calibration curves of Amadori-PE (A) and PE (B) from quantitative analysis by LC-MS/MS with MRM. The calibration curves were constructed with Amadori-PE and PE standards of different concentrations (0.1–100 pmol/injection).

pounds. However, the characteristics and quantitative method of Amadori-PE have not been established. Therefore, the objectives of the present study were to develop an analytical method of Amadori-PE in biological samples and then to prove the occurrence of Amadori-PE in vivo.

Recent studies reported the detection of Amadori-PE in biological samples (i.e., blood plasma, red blood cells, and human atherosclerotic plaques) using LC-MS (25–28). During LC-MS analysis, chromatographic peaks that showed mass increments of 162 Da (most likely $C_6H_{10}O_5$) relative to the molecular masses of nonglycated PE were tentatively ascribed to Amadori-PE. However, this identification is not quite complete, because of the lack of structural information regarding Amadori-PE (i.e., glycated polar head group and fatty acid moieties). To overcome this problem, in this study, we applied the QTRAP for the analysis of lipid glycation products. The QTRAP combines all of the functions of a classical triple quadrupole mass spectrometer with the additional capabilities of a QqLIT mass spectrometer (12). As a result, we demonstrate that the QTRAP is particularly suitable for the analysis of Amadori-PE in biological samples. Neutral loss scanning is highly effective to "fish out" Amadori-PE from human plasma. Product ion scanning also provides reliable structural information about Amadori-PE. In addition, LC-MS/MS with MRM permits quantitative determination of the predominant plasma Amadori-PE species.

In qualitative analysis, we discovered that protonated Amadori-PE is liable to generate an abundant product ion of $[M+H-303]^+$ in the collision cell. When the QTRAP was used to perform neutral loss (303 Da) scanning, only Amadori-PE species were detectable. Normally, it is very difficult to detect Amadori-PE in biological samples because of overlapping background contaminants (25). In contrast, by means of neutral loss scanning, individual Amadori-PE species in plasma could be effectively identified without any preliminary LC separations. On the other hand, Ravandi et al. (25) performed LC-MS analysis of Amadori-PE in diabetic plasma and compared the glycated species of the diacyl PE and alkenyl-acyl PE (plasmalogen). They suggested that only the diacyl PE species, not the alkenyl-acyl species, became glycated. However, the Amadori products of both diacyl and plasmalogenic species were clearly detected by means of the present neutral loss scan. The results in Fig. 3 show that diacyl and alkenyl-acyl PE are randomly glycated in vivo, but ion intensities of Amadori-PE species do not show their relative amounts (29). Alkenyl-acyl PE (plasmalogen) is widely distributed in most mammalian cells and tissues (30), and it has attracted much attention as an endogenous antioxidant in protecting cell membranes and lipoproteins from reactive oxygen species (31). Because Amadori-PE generates reactive oxygen species (7), the glycation of plasmalogen PE may lose its antioxidant property, thus eventually increasing oxidative stress in vivo. Because phosphatidylserine (PS) also has an amino group, the glycation of PS may occur in biological materials. However, in this study, glycated PS was undetectable in human plasma (data not shown). Similarly, Ravandi et al. (25) were also unable to detect glycated PS in human plasma or red blood cell membranes.

In quantitative analysis, LC-MS/MS with MRM is highly useful for the determination of Amadori-PE in human plasma. Synthetic Amadori-PE standards (16:0-18:1, 16:0-20:4, and 16:0-22:6) permitted the calculation of the corresponding endogenous Amadori-PE levels, which are predominantly present in human plasma. Internal standard (nonnaturally occurring 18:1-18:1 Amadori-PE) is necessary to cope with possible variations in the extraction yield of Amadori-PE from plasma. We were unable to quantify plasmalogen-type Amadori-PE because of a lack of standards. According to the detection of Amadori-PE, MRM was ~100 times more sensitive than neutral loss scanning because of the specific ability of MRM to target molecular ions (32, 33). We used a reverse-phase column, because it has often been used for the separation of the molecular species of phospholipids (34). Under optimized conditions, Amadori-PE standards could be clearly separated on MRM chromatograms. The calibration curves were linear over a wide concentration range. The detection limit of Amadori-PE was ~0.1 pmol/injection. Generally, the LC detection limit of phospholipid-derived chemical mediators, such

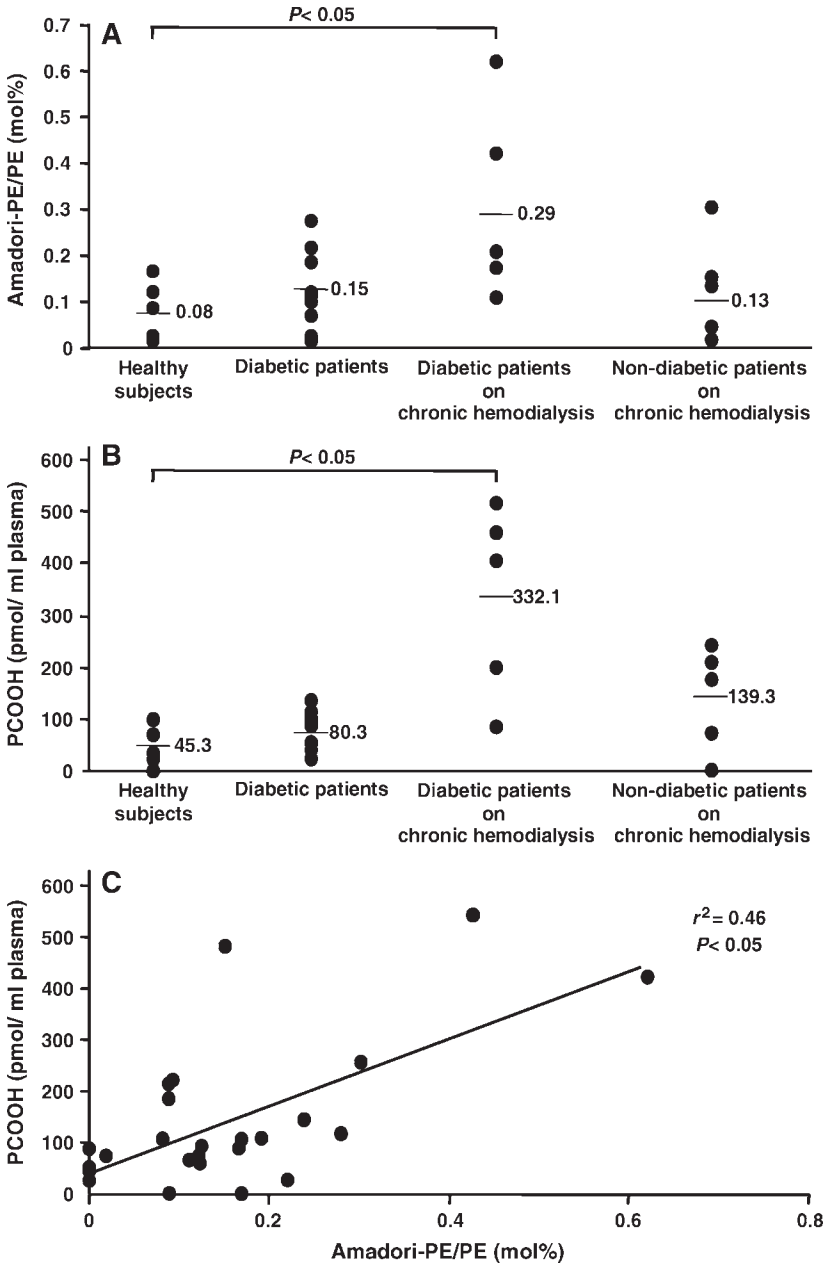

Fig. 7. Amadori-PE (A), phosphatidylcholine hydroperoxide (PCOOH; B), and their correlation (C) in plasma of healthy subjects, diabetic patients, diabetic patients on chronic hemodialysis, and nondiabetic patients on chronic hemodialysis.

as phospholipid hydroperoxides (2, 35) and platelet-activating factor-like phospholipids (36), is above picomole levels. Hence, the sensitivity of the present LC-MS/MS with MRM for Amadori-PE is relatively high. As shown in Fig. 6, LC-MS/MS with MRM could be applied to the determination of Amadori-PE in plasma. The relative amount of Amadori-PE to PE in plasma of diabetic patients (0.15 mol%) and of diabetic patients on chronic hemodialysis (0.29 mol%) was higher than that of healthy subjects (0.08 mol%). From this result, we could confirm that membrane lipid glycation increases along with diabetic duration. The reason for the higher Amadori-PE in plasma of diabetic patients on chronic hemodialysis compared with diabetic patients remains unclear. For protein glycation, it is reported that AGE is accumulated in plasma of diabetic as well as nondiabetic patients with renal disease (37). Other reports showed that kidney transplantation decreases plasma AGE (38). Considering our results and previous reports (37, 38), it appears that, like protein AGE, plasma Amadori-PE may be a useful marker in renal disease associated with diabetes. On the other hand, Amadori-PE levels in nondiabetic patients on chronic hemodialysis were slightly higher than those of control subjects. Phospholipid concentration in nondiabetic patients on chronic hemodialysis (6.3 mg/dl) was significantly higher than that of control subjects (5.2 mg/dl) and diabetic patients (5.6 mg/

dl). Therefore, it is likely that lipid glycation is affected not only by hyperglycemia (glucose) but also by hyperlipidemia (phospholipids) (3).

As mentioned in the introduction, we previously confirmed that plasma PCOOH increases in type 2 diabetes patients and that the concentration is proportional to Hb_{A1c} (4). Our subsequent study demonstrated that Amadori-PE is capable of reactive oxygen species generation and thereby triggers lipid peroxidation (7). We also recently demonstrated that Amadori-PE is an important compound that promotes vascular disease as a result of its angiogenic activity on endothelial cells (8). In this study, the positive correlation between plasma Amadori-PE and PCOOH was indeed observed in vivo (Fig. 6). Generally, PCOOH is the predominant hydroperoxide present in plasma of healthy subjects, and plasma PEOOH content is lower (2). However, in this study, both PCOOH and PEOOH were detected as major types of hydroperoxides in plasma of some diabetic patients (data not shown). Therefore, it is likely that Amadori-PE is involved in PE susceptibility to peroxidation. These results suggested that under hyperglycemic conditions, PE is exposed to glycation, yielding Amadori-PE in the blood, which causes oxidative stress and angiogenic effects. Consequently, lipid glycation and lipid peroxidation may play active parts in the development of diabetes. To further understand the involvement of lipid glycation in the pathophysiology of human disease, we are now using QTRAP LC-MS/MS to quantify Amadori-PE in blood and tissue samples from healthy and nonhealthy humans. In addition, based on our method, it is feasible to develop new analytical means to determine lipid glycation products other than Amadori-PE.

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