

Aminophospholipid glycation and its inhibitor screening system: a new role of pyridoxal 5'-phosphate as the inhibitor

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Abstract Peroxidized phospholipid-mediated cytotoxicity is involved in the pathophysiology of a number of diseases [i.e., the abnormal increase of phosphatidylcholine hydroperoxide (PCOOH) found in the plasma of type 2 diabetic patients]. The PCOOH accumulation may relate to Amadori-glycated phosphatidylethanolamine (deoxy-D-fructosyl PE, or Amadori-PE), because Amadori-PE causes oxidative stress. However, lipid glycation inhibitor has not been discovered yet because of the lack of a lipid glycation model useful for inhibitor screening. We optimized and developed a lipid glycation model considering various reaction conditions (glucose concentration, temperature, buffer type, and pH) between PE and glucose. Using the developed model, various protein glycation inhibitors (aminoguanidine, pyridoxamine, and carnosine), antioxidants (ascorbic acid, α -tocopherol, quercetin, and rutin), and other food compounds (L-lysine, L-cysteine, pyridoxine, pyridoxal, and pyridoxal 5'-phosphate) were evaluated for their antiglycative properties. Pyridoxal 5'-phosphate and pyridoxal (vitamin B₆ derivatives) were the most effective antiglycative compounds. These pyridoxals could easily be condensed with PE before the glucose/PE reaction occurred. Because PE-pyridoxal 5'-phosphate adduct was detectable in human red blood cells and the increased plasma Amadori-PE concentration in streptozotocin-induced diabetic rats was decreased by dietary supplementation of pyridoxal 5'-phosphate, it is likely that pyridoxal 5'-phosphate acts as a lipid glycation inhibitor *in vivo*, which possibly contributes to diabetes prevention.—Higuchi, O., K. Nakagawa, T. Tsuzuki, T. Suzuki, S. Oikawa, and T. Miyazawa. **Aminophospholipid glycation and its inhibitor screening system: a new role of pyridoxal 5'-phosphate as the inhibitor.** *J. Lipid Res.* 2006. 47: 964–974.

Supplementary key words lipid glycation inhibitor • phosphatidylethanolamine • diabetes

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

Recently, while investigating why PCOOH increases in diabetic plasma, we found that diabetic plasma contained an abnormal amount of glycated lipid. The glycated lipid was identified as an Amadori product of phosphatidylethanolamine (deoxy-D-fructosyl phosphatidylethanolamine, or Amadori-PE) (Fig. 1) by LC online with hybrid quadrupole/linear ion-trap mass spectrometry (QTRAP LC/MS/MS) (5, 6). Amadori-PE generates reactive oxygen species and thereby triggers lipid peroxidation (7). 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) (5, 6), leading to a disorder of cellular integrity (i.e., angiogenesis stimulation) (8). Amadori-PE and PCOOH could play a role in the development of diabetes. Several foods (i.e., infant formula and chocolate) contain high amounts of Amadori-PE (9). In these products, lipid glycation could impair food flavor, color, and nutritive value.

Abbreviations: AGE, advanced glycation end product; dioleoyl-PE, 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine; ELSD, evaporative light-scattering detection; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; QTRAP LC/MS/MS, quadrupole/linear ion-trap liquid chromatography tandem mass spectrometry; RBC, red blood cell; STZ, streptozotocin.

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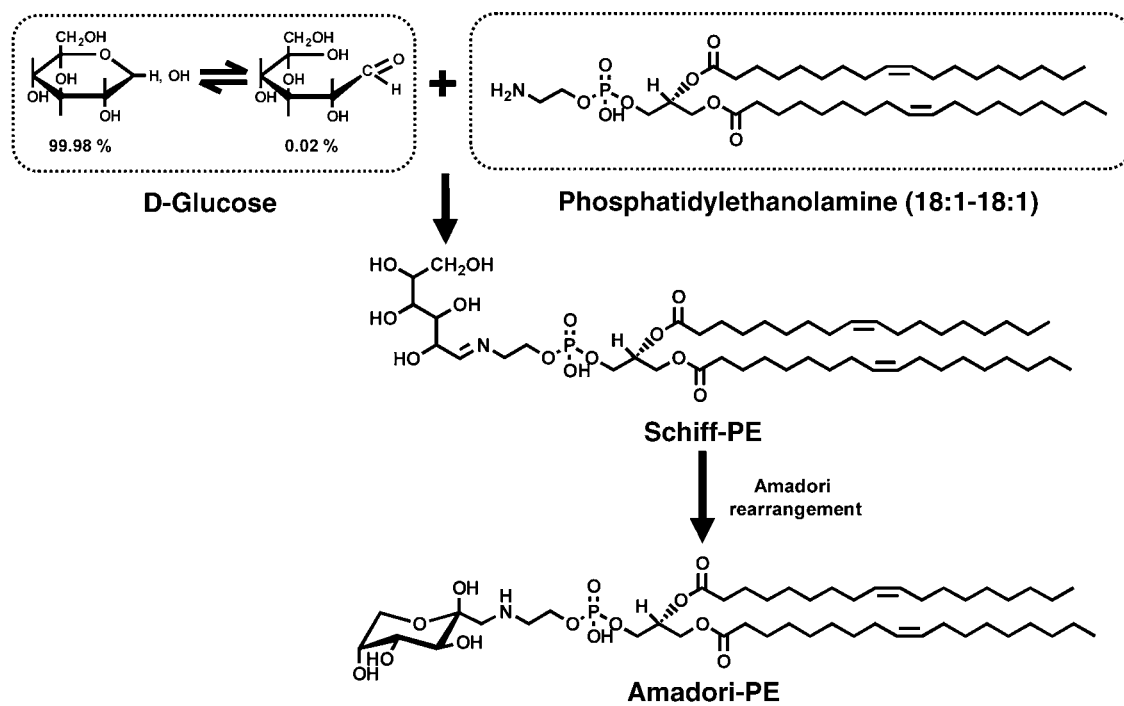


Fig. 1. Scheme for the glycation of phosphatidylethanolamine (PE). Glucose reacts with the amino group of PE to form Schiff-PE, which undergoes an Amadori rearrangement to yield Amadori-PE (deoxy-D-fructosyl PE).

Despite the potential significance of Amadori-PE in pathological signaling and food deterioration, lipid glycation inhibitor has not yet been discovered. This is because of the lack of a lipid glycation model useful for inhibitor screening. PE glycation involves a Schiff base formation and its rearrangement to Amadori-PE. Therefore, to develop a lipid glycation model, it is necessary to elucidate the optimal reaction conditions (glucose concentration, temperature, buffer type, and pH) for Schiff-PE and Amadori-PE formation.

In this work, we optimized reaction conditions between PE and glucose and developed a lipid glycation model. Using this model, we found that pyridoxal 5'-phosphate and pyridoxal (vitamin B₆ derivatives) are attractive anti-glycative compounds. The possible therapeutic roles of vitamin B₆ derivatives *in vivo* were evaluated.

MATERIALS AND METHODS

Materials

1,2-Di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (dioleoyl-PE), ascorbic acid, α -tocopherol, L-cysteine hydrochloride monohydrate, L-lysine dihydrochloride, L-carnosine, quercetin, and rutin were purchased from Wako (Kyoto, Japan). Ammonium acetate, aminoguanidine, and streptozotocin (STZ) were obtained from Sigma (St. Louis, MO). Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride, and pyridoxal 5'-phosphate were from TCI (Tokyo, Japan). Schiff-PE and Amadori-PE standards were prepared from Maillard reaction products between dioleoyl-PE and D-glucose by HPLC, as described previously (7). All other reagents were of analytical grade.

Lipid glycation model

The stock buffer solutions (0.1 M acetate buffer, pH 4.0–5.5, 0.1 M phosphate buffer, pH 6.0–8.0, 0.1 M Tris-HCl buffer, pH 7.5–9.0, and 0.1 M sodium carbonate buffer, pH 9.5–10.0) were prepared. Solutions 1–3 were prepared before use. For solution 1 (dioleoyl-PE solution), 3 mM dioleoyl-PE was dissolved in methanol; for solution 2 (glucose solution), 0.5–2.5 M D-glucose was dissolved in stock buffer; and for solution 3 (working buffer solution), the buffer was prepared by diluting stock buffer with methanol at a ratio of 3:4, 2:5, 1:6, or 0:7 (v/v). To initiate lipid glycation, dioleoyl-PE solution (100 μ l of solution 1), D-glucose solution (200 μ l of solution 2), and working buffer solution (700 μ l of solution 3) were mixed. The mixture was incubated at 37–60°C for 0–48 h under an air atmosphere. After incubation, to extract lipid-soluble products, chloroform, methanol, and water were added to the incubated mixture so that the final ratio of chloroform-methanol-water was 2:1:1 (10). After centrifugation at 1,600 g for 10 min, the chloroform layer (lipid fraction) was collected and evaporated. The lipid residue was dissolved in 1 ml of methanol, and a portion (50 μ l) was subjected to LC coupled to an evaporative light-scattering detection (ELSD) device to monitor the yield of Schiff-PE and Amadori-PE, as described below.

Lipid glycation inhibitor

Dioleoyl-PE (3 mM) in methanol and D-glucose (2.5 M) in phosphate buffer (0.1 M, pH 7.4) were prepared. Test samples (ascorbic acid, L-cysteine, L-lysine, acetylsalicylic acid, L-carnosine, quercetin, rutin, pyridoxine, pyridoxamine, pyridoxal, or pyridoxal 5'-phosphate, each 1, 5, and 10 mM) in phosphate buffer and α -tocopherol (10 mM) in methanol were prepared. Dioleoyl-PE solution (100 μ l), D-glucose solution (200 μ l), and methanol (600 μ l) were mixed, and test sample (100 μ l) was added. The mixture was incubated at 37°C for 0–48 h under an air atmo-

sphere. At different time intervals (2 and 48 h), Schiff-PE and Amadori-PE were extracted and determined by LC-ELSD.

LC-ELSD analysis

An ODS column (TSK gel ODS-80-Ts, 4.6×150 mm; Tosoh, Tokyo, Japan) was used with a mobile phase of methanol-water (99:1) containing 5 mM ammonium acetate at a flow rate of 1 ml/min. The column temperature was maintained at 35°C. At the postcolumn, the eluent was split. One of the split eluents (flow rate, 0.95 ml/min) was sent to a Sedex 55 ELSD device (Sedere, Alfortville, France). The temperature of the drift tube was set at 60°C. Nitrogen gas was used as nebulizer gas at a pressure of 2.0 bar, and the gain was set at 8. Peak areas of Schiff-PE (5.6 min), Amadori-PE (7.5 min), and dioleoyl-PE (9.2 min) were recorded using a Chromatocorder 12 (System Instruments, Tokyo, Japan). Schiff-PE, Amadori-PE, and dioleoyl-PE concentrations were calculated based on their calibration curves.

To identify Schiff-PE and Amadori-PE, the other eluent (flow rate, 0.05 ml/min) was sent to a Mariner electrospray ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Mass spectrometry (MS) was carried out in the positive ion measurement mode with spray voltage of 2,900 V, nozzle potential of 220 V, and nozzle temperature of 140°C. The flow rate of nebulizer gas was 0.3 ml/min. Full-scan spectra were obtained by scanning masses between m/z 400 and 1,000 at 3 s/scan.

Extraction of PE-pyridoxal 5'-phosphate adduct from human red blood cells

Healthy male volunteers (mean age, 24 years; $n = 3$) participated in this study. Written informed consent was obtained from all volunteers. Blood (20 ml) was collected from all subjects into tubes containing 0.1% EDTA-2Na as anticoagulant and then subjected to centrifugation at 1,000 g for 15 min at 4°C. After plasma and buffy coat were removed, red blood cells (RBCs) were washed three times with phosphate-buffered saline (pH 7.4) to prepare packed cells. RBC total lipids were extracted from packed cells (1 ml) with 2-propanol/chloroform by the method of Rose and Oklander (11). The lipid extract was redissolved in 1 ml of methanol.

To reduce PE-pyridoxal 5'-phosphate adduct present in RBCs, solid NaBH_4 (1 mmol) was added to the RBC lipid extract (1 ml). After incubation at 4°C for 1 h, the mixture was evaporated and dissolved in 4 ml of chloroform. The chloroform solution was washed with water to remove NaBH_4 , evaporated, and finally dissolved in 1 ml of methanol-water (99:1; containing 5 mM ammonium acetate). A portion (10 μl) was subjected to QTRAP LC/MS/MS, as described below.

Analysis of PE-pyridoxal 5'-phosphate adduct

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 hybrid quadrupole/linear ion trap mass spectrometer (6).

Neutral loss scan was used for the qualitative analysis of the reduced form of PE-pyridoxal 5'-phosphate adduct. Standard PE-pyridoxal 5'-phosphate adduct (reduced form) was synthesized from dioleoyl-PE and pyridoxal 5'-phosphate, according to a previous report (12). Either RBC extract (100 μl) or standard (10 pmol/100 μl) was infused directly to the QTRAP by a flow

syringe pump at 0.01 ml/min. Positive ionization mode was adopted. QTRAP parameters, including collision energy and spraying conditions, were optimized with standard (dioleoyl-PE-pyridoxal 5'-phosphate). The QTRAP was programmed to scan the protonated molecules that had a neutral loss of 372 Da after collision-activated dissociation at the collision cell (Q2; collision gas, N_2 , 4 p.s.i.; 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 as the turbo, nebulizer, and curtain gas were set at 70, 50, and 20 p.s.i., respectively.

Multiple reaction monitoring (MRM) was used for the detection of predominant molecular species of PE-pyridoxal 5'-phosphate adduct in RBCs. Briefly, RBC extract (10 μl) was separated on a TSK gel ODS 100s (2.0 mm \times 150 mm; Tosoh) fitted with a

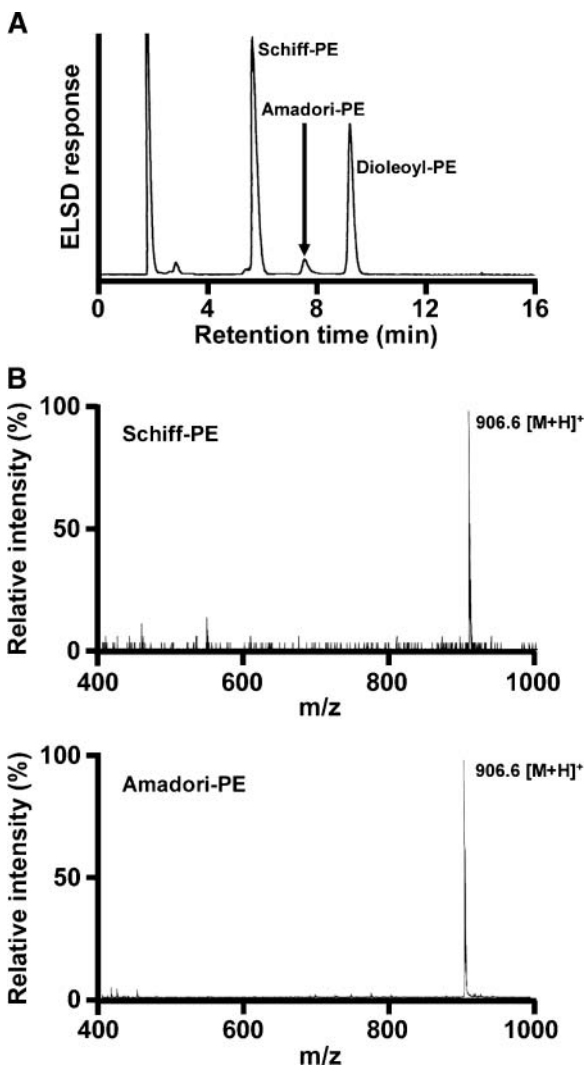


Fig. 2. Production of Schiff-PE and Amadori-PE during glycation. 1,2-Di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (dioleoyl-PE; 0.3 mM) was incubated with D-glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) at 37°C for 24 h. Lipid-soluble products were extracted from the incubation mixture by the method of Folch, Lees, and Sloane-Stanley (10). The lipid extract was dissolved in 1 ml of methanol, and a portion (50 μl) was subjected to liquid chromatography-evaporative light-scattering detection-mass spectrometry (LC-ELSD-MS). A: ELSD chromatogram. B: Mass spectra of the peaks (5.6 and 7.5 min) detected in chromatogram A.

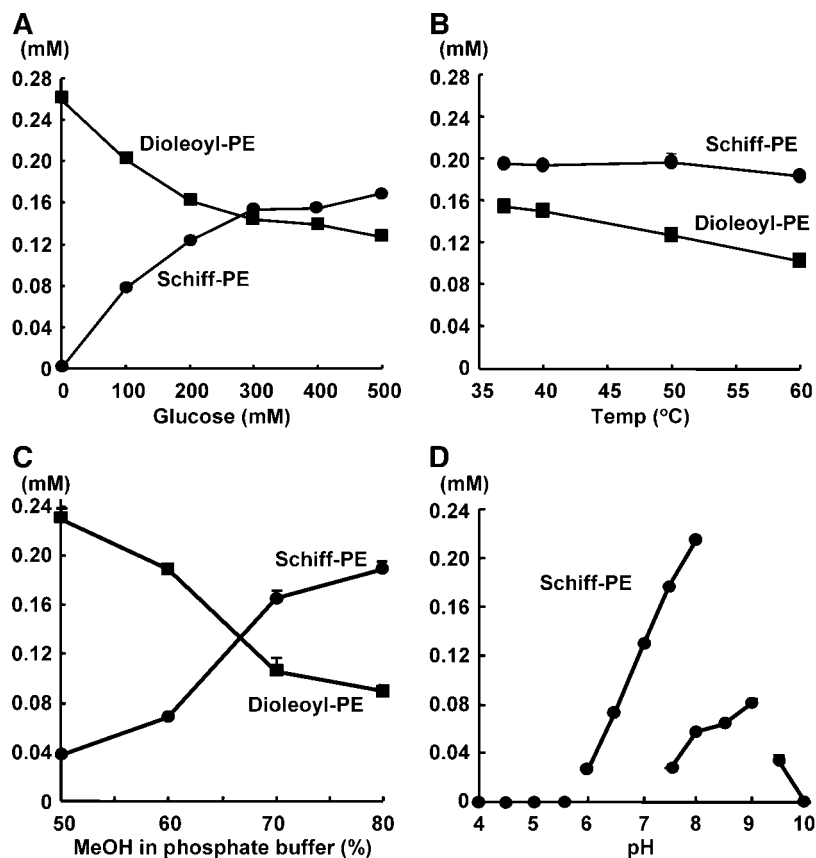


Fig. 3. Effect of incubation conditions [glucose (A), temperature (B), incubation solvent (C), and pH (D)] on lipid glycation kinetics. Dioloeyl-PE (0.3 mM) was incubated with α -glucose (0–500 mM) in 1 ml of 50–80% methanolic buffer (0.1 M, pH 4.0–10.0) at 37–60°C for 2 h. After incubation, Schiff-PE and dioloeyl-PE were determined by LC-ELSD. Values are means \pm SD ($n = 3$). MeOH, methanol.

guard column (Inertsil ODS-3, 3.0 mm \times 10 mm; GL Science, Tokyo, Japan). The column was eluted with a mixture of methanol-water (99:1; containing 5 mM ammonium acetate) at a flow rate of 0.3 ml/min, and the column temperature was maintained at 30°C. At the postcolumn, predominant molecular species of PE-pyridoxal 5'-phosphate adduct in RBCs were individually detected by the QTRAP using MRM for the transition of parent ion to product ion. The collision energy for the transition was 30 eV. The dwell time for the transition was 100 ms. Other QTRAP conditions were as described above.

Animal study

Four week old male Sprague-Dawley rats weighing 70–80 g (CLEA, Tokyo, Japan) were used. Rats were given free access to standard laboratory chow (MF pellet; Oriental Yeast, Tokyo, Japan) and water for 1 week before the experiment began. Rats were housed in a temperature- and humidity-controlled room with a 12 h light cycle. The study was conducted in accordance with the Animal Experimentation Guidelines of Tohoku University.

Diabetes was induced in rats by a single intraperitoneal injection of 80 mg/kg STZ dissolved in 0.1 M sodium citrate buffer (pH 4.5), whereas control rats received an injection of citrate buffer. Blood glucose was measured 3 days after STZ injection. Rats with blood glucose levels of ≥ 15 mM were considered diabetic and were used as diabetic rats in the study.

One week after the administration of STZ and/or citrate buffer, rats were divided into four groups: normal rats (control; $n = 6$), normal rats receiving 2 mM aqueous pyridoxal 5'-phosphate (equivalent to 300 mg/kg body weight/day; $n = 6$), diabetic STZ rats ($n = 6$), and diabetic rats receiving 2 mM aqueous pyridoxal 5'-phosphate ($n = 6$). After feeding for 10 weeks, rats were fasted for 12 h and anesthetized with diethyl ether, and blood plasma samples were collected.

Plasma Amadori-PE concentration was determined by QTRAP LC-MS/MS (5, 6). Plasma PCOOH was quantified by chemiluminescence detection-LC (2). Fasting blood glucose, cholesterol, and triglyceride in plasma were measured enzymatically with commercial kits supplied by Wako (Kyoto, Japan).

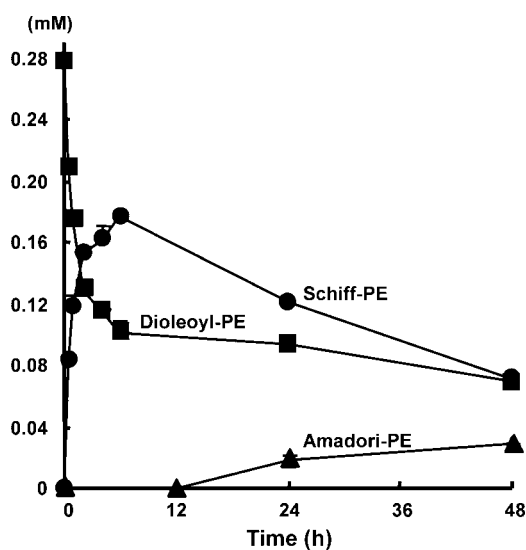


Fig. 4. Time course of changes in the yields of lipid glycation products under optimal incubation conditions. Dioloeyl-PE (0.3 mM) was incubated with α -glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) at 37°C for 48 h. At different time intervals, Schiff-PE, Amadori-PE, and dioloeyl-PE were determined by LC-ELSD. Values are means \pm SD ($n = 3$).

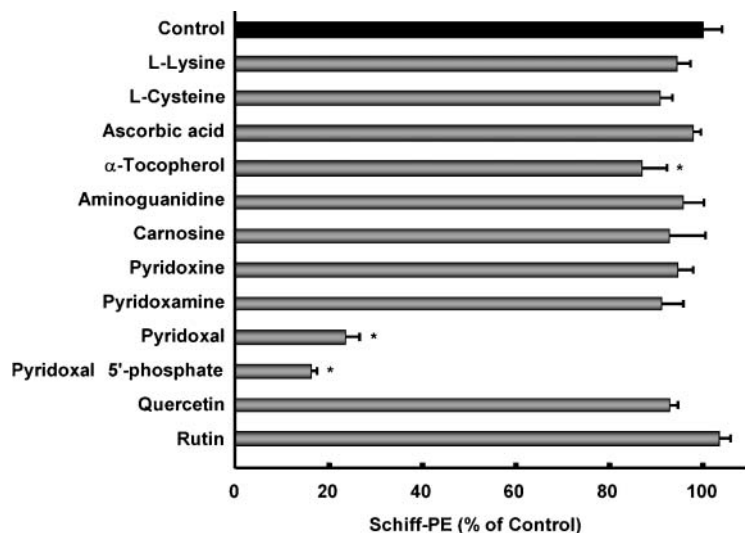


Fig. 5. Screening for lipid glycation inhibitor. Dioleoyl-PE (0.3 mM) was incubated with D-glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) in the absence (control) or presence of test compound (1 mM) at 37°C for 2 h. After incubation, Schiff-PE was determined by LC-ELSD. * Significant difference compared with control ($P < 0.05$). Values are means \pm SD ($n = 3$).

Statistical analysis

The data are expressed as means \pm SD. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni posthoc test. $P < 0.05$ was accepted as significant.

RESULTS

Development of the lipid glycation model system

Figure 2 shows a typical ELSD chromatogram when glucose-treated dioleoyl-PE in methanolic phosphate buffer was subjected to LC-ELSD. After glycation, dioleoyl-PE itself (9.2 min) was decreased, and two peak components (5.6 and 7.5 min) appeared as the major lipid glycation products. Despite the same molecular ion $[M + H]^+$ at m/z 906.6, these peaks could be identified as Schiff-PE (5.6 min) and Amadori-PE (7.5 min) based on a comparison of retention times with standards and the reactivity of reducing agents (i.e., NaBH_3CN or NaBH_4) (data not shown).

As shown in Fig. 3, lipid glycation kinetics depended closely on incubation conditions. High glucose concentration (300–500 mM), neutral pH (7.0–7.4), and methanolic phosphate buffer (70–80% methanol) all enhanced Schiff-PE formation, whereas reaction temperature did not.

Based on these results, we chose the best lipid glycation system: dioleoyl-PE (0.3 mM) was incubated with glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) at 37°C. In this system, Schiff-PE formation reached its maximum level (0.18 mM) at 6 h after incubation and then decreased gradually (Fig. 4). During 12–48 h of incubation, Amadori-PE formation occurred consistently. These findings are well rationalized by the Maillard theory: PE reacts with glucose to form Schiff-PE, followed by rearrangement to Amadori-PE. Therefore, if Schiff-PE formation can be minimized, the responsible compound may be used as a lipid glycation inhibitor.

Inhibition of lipid glycation by pyridoxal 5'-phosphate and pyridoxal

Using this lipid glycation system, we investigated the inhibitory effects of several compounds (ascorbic acid, α -tocopherol, L-lysine, L-cysteine, aminoguanidine, carnosine, pyridoxine, pyridoxamine, pyridoxal, pyridoxal 5'-phosphate, quercetin, and rutin, each at 1 mM) on Schiff-PE formation (Fig. 5). Pyridoxal 5'-phosphate and pyridoxal (vitamin B₆ derivatives) were screened out as the most attractive antiglycative compounds. Both pyridoxal 5'-phosphate and pyridoxal inhibited Schiff-PE formation in a dose-dependent manner and thereby prevented Amadori-PE production (Fig. 6).

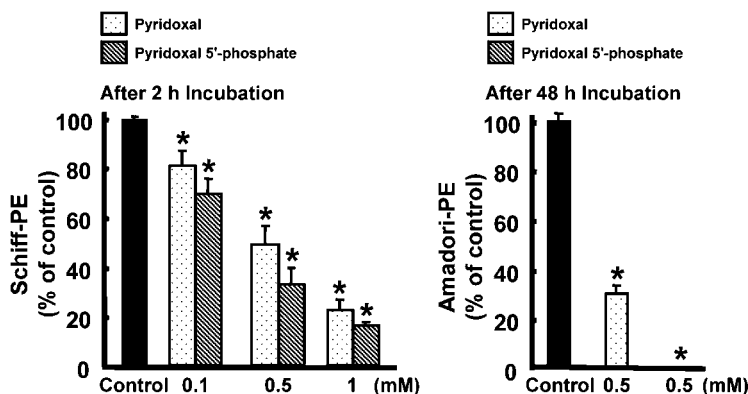


Fig. 6. Inhibitory effect of pyridoxal 5'-phosphate and pyridoxal on lipid glycation. Dioleoyl-PE (0.3 mM) was incubated with D-glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) in the absence (control) or presence of pyridoxal 5'-phosphate or pyridoxal (each 0.1–1 mM) at 37°C for 2 and 48 h. * Significant difference compared with control ($P < 0.05$). Values are means \pm SD ($n = 3$).

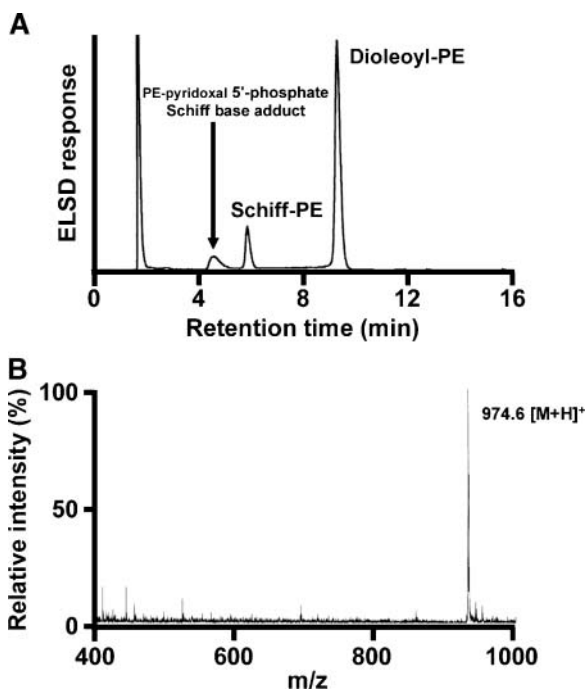


Fig. 7. Typical ELSD chromatogram of the pyridoxal 5'-phosphate/dioleoyl-PE/glucose system. Dioleoyl-PE (0.3 mM) was incubated with D-glucose (500 mM) in 1 ml of 70% methanolic phosphate buffer (0.1 M, pH 7.4) in the presence of pyridoxal 5'-phosphate (1 mM) at 37°C for 2 h. Lipid-soluble products were extracted from the incubation mixture by the method of Folch, Lees, and Sloane-Stanley (10) and subjected to LC-ELSD-MS. A: ELSD chromatogram. B: Mass spectrum of the peak (4.6 min) detected in chromatogram A.

Figure 7 shows a typical ELSD chromatogram of the pyridoxal 5'-phosphate/dioleoyl-PE/glucose system. Inter-

estingly, an unknown peak (4.6 min) appeared. This peak was identical to PE-pyridoxal 5'-phosphate Schiff base adduct ($[M + H]^+$, m/z 974.6) based on its MS profile. In the pyridoxal/dioleoyl-PE/glucose system, PE-pyridoxal Schiff base adduct ($[M + H]^+$, m/z 893.6) was formed (data not shown). Therefore, it is likely that pyridoxal 5'-phosphate and pyridoxal can easily condense with dioleoyl-PE, thereby competitively inhibiting lipid glycation (**Fig. 8**).

Occurrence of PE-pyridoxal 5'-phosphate adduct in human RBCs

Despite the wide distribution of pyridoxal 5'-phosphate in human tissues and body fluids (13), the occurrence of PE-pyridoxal 5'-phosphate adduct in vivo has never been investigated. As shown in **Fig. 9**, in positive ion mode, collision-induced dissociation of a reduced form of PE-pyridoxal 5'-phosphate adduct produced a diglyceride ion ($[M + H - 372]^+$) permitting neutral loss scanning and MRM. When NaBH_4 -treated RBC extract was infused into the QTRAP, molecular species of PE-pyridoxal 5'-phosphate adduct (reduced forms) could be screened out by neutral loss scanning (**Fig. 10**). It was found that the predominant molecular species of PE-pyridoxal 5'-phosphate adduct in RBCs were 16:0-18:1 (m/z 949.9), 16:0-18:2 (m/z 947.9), 16:0-20:4 (m/z 971.9), 16:0-22:6 (m/z 995.9), 18:0-18:2 (m/z 975.9), 18:0-20:4 (m/z 999.9), and 18:0-22:6 (m/z 1,023.8) as diacyl species and 16:0-20:4 (m/z 955.8), 16:0-22:6 (m/z 1,007.8), 18:0-20:4 (m/z 983.8), 18:0-22:6 (m/z 1,007.8), and 18:1-20:4 (m/z 981.9) as alkenyl-acyl species (plasmalogen). The neutral loss spectra of PE-pyridoxal 5'-phosphate adduct and PE itself indicated that the molecular species of PE were randomly condensed with pyridoxal 5'-phosphate. These results provided the

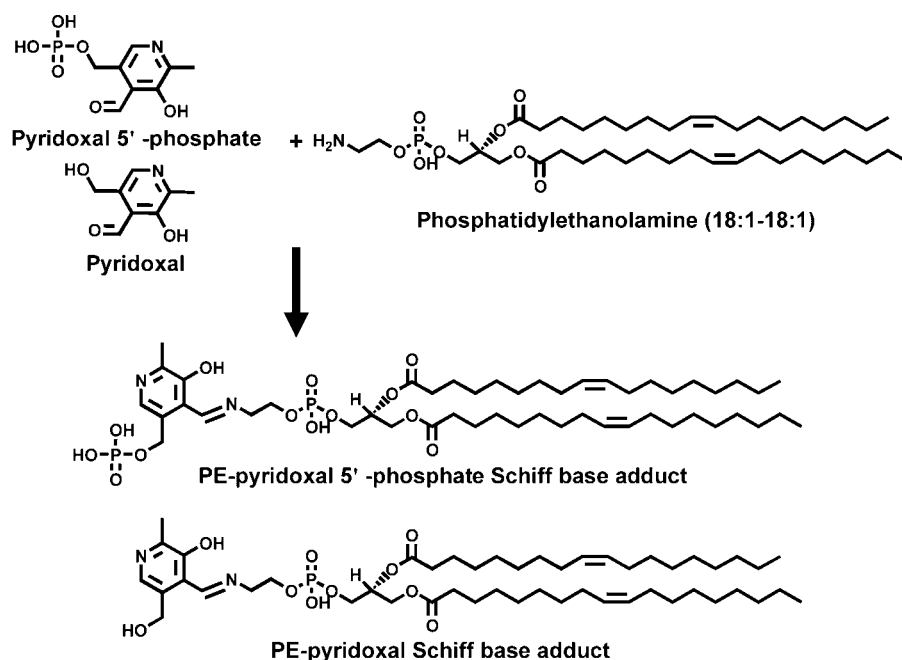


Fig. 8. Scheme for the condensation of pyridoxal 5'-phosphate or pyridoxal with PE. Pyridoxal 5'-phosphate or pyridoxal reacts with the amino group of PE to form Schiff base adduct.

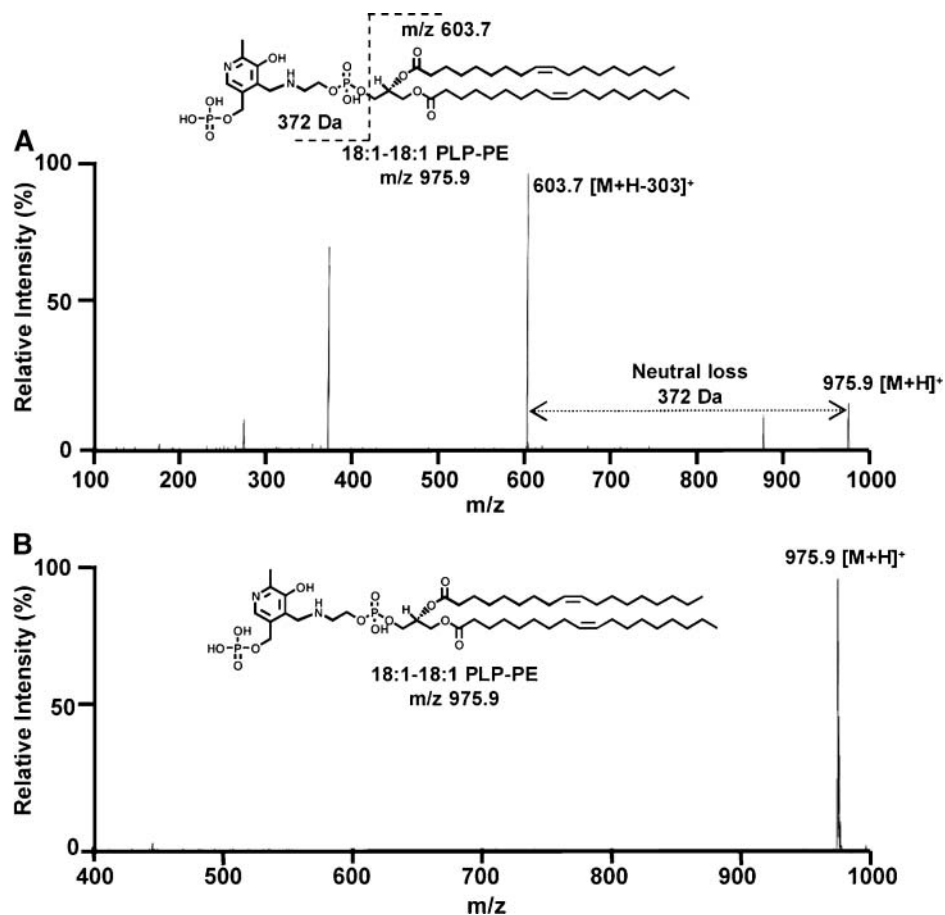


Fig. 9. Specific detection of a reduced form of PE-pyridoxal 5'-phosphate adduct using the QTRAP mass spectrometer. A: Product ion spectrum of the $[M + H]^+$ ion at m/z 975.9 of 18:1-18:1-PE-pyridoxal 5'-phosphate adduct (reduced form). B: Neutral loss scan for 372 Da showing specific detection of 18:1-18:1-PE-pyridoxal 5'-phosphate adduct (reduced form). The standard solution of 18:1-18:1-PE-pyridoxal 5'-phosphate adduct (reduced form) was infused directly into the QTRAP using a syringe pump at a flow rate of 0.01 ml/min.

first direct structural evidence for the existence of PE-pyridoxal 5'-phosphate adduct *in vivo*.

Interfacing LC with the QTRAP enabled the separation and detection of predominant molecular species of PE-pyridoxal 5'-phosphate adduct (reduced forms) in RBCs (**Fig. 11**). The concentration of PE-pyridoxal 5'-phosphate adduct [16:0-18:2 (m/z 947.6), one of the most predominant molecular species] was $\sim 78 \pm 12$ pmol/ml packed cells. In contrast, the concentration of PE-pyridoxal adduct was below the detection limit (<1 pmol/ml packed cells). These findings suggest a new role of pyridoxal 5'-phosphate in preventing lipid glycation *in vivo*.

Effect of dietary pyridoxal 5'-phosphate on lipid glycation *in vivo*

In this study, the effect of dietary pyridoxal 5'-phosphate on lipid glycation was investigated using STZ-induced diabetic rats. As shown in **Fig. 12**, the amount of Amadori-PE (18:0-22:6-Amadori-PE, a predominant species in plasma) in control rat plasma (STZ-untreated; $n = 6$) was 44.5 ± 25.7 pmol/ml. In STZ-induced diabetic rats (80 mg/kg body weight; $n = 6$), a higher concentration of plasma Amadori-PE (18:0-22:6-Amadori-PE) was seen

(945.7 ± 429.0 pmol/ml). Plasma Amadori-PE concentration was proportional to plasma PCOOH (a marker of oxidative stress; control rats, 8.6 ± 2.8 pmol/ml; STZ rats, 68.9 ± 33.7 pmol/ml). The increments of both plasma Amadori-PE and PCOOH were significantly suppressed by 38% and 39%, respectively, by dietary supplementation of 2 mM pyridoxal 5'-phosphate (300 mg/kg body weight/day) to STZ rats for 10 weeks. Although no significant difference was observed in plasma lipids between STZ rats and STZ rats fed pyridoxal 5'-phosphate, dietary pyridoxal 5'-phosphate tended to improve the plasma cholesterol and triglyceride of STZ rats (**Fig. 13**).

DISCUSSION

Protein nonenzymatically reacts with glucose, leading to the production of Schiff base and ultimately Amadori product. These early glycation products are further transformed into advanced glycation end products (AGEs) (14). Numerous studies have described the formation and accumulation of Amadori products, such as glycated

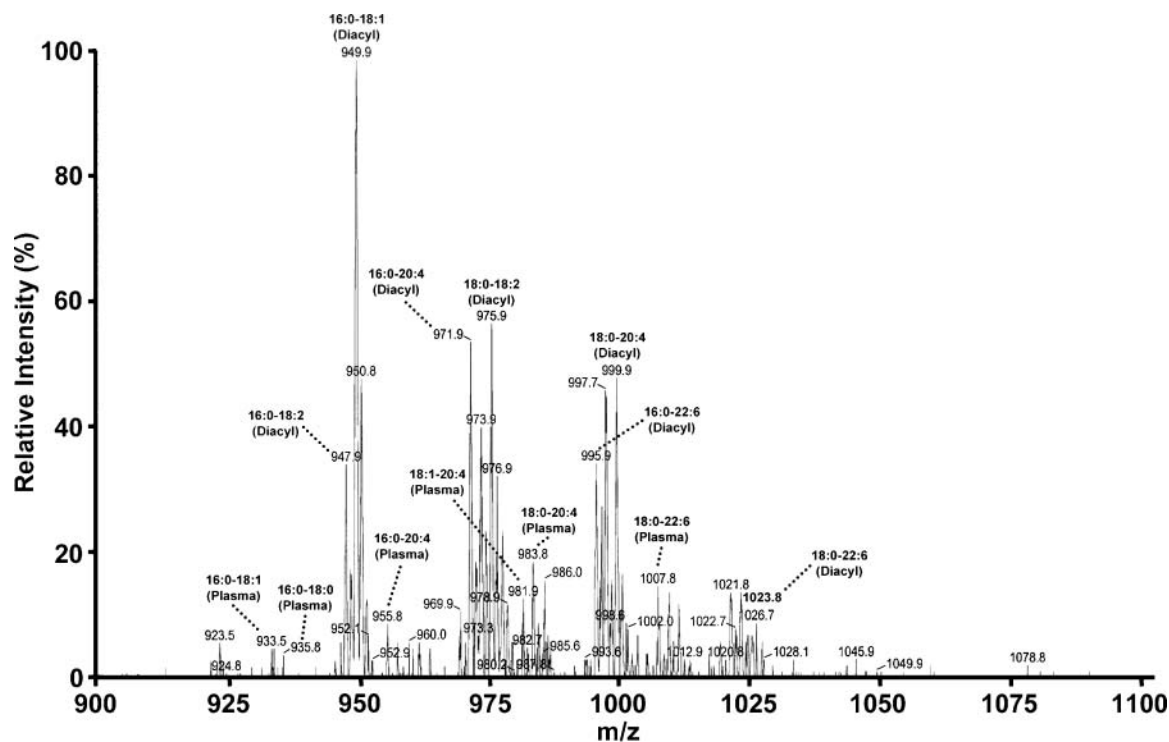


Fig. 10. Neutral loss scan spectra of human red blood cell (RBC) extract. Neutral loss of 372 Da showing the specific detection of molecular species of PE-pyridoxal 5'-phosphate adduct (reduced form) in RBCs. Total lipids were extracted from human RBCs (packed cells) by the method of Rose and Oklander (11). RBC lipid extract was treated with NaBH_4 and infused into the QTRAP using a syringe pump at a flow rate of 0.01 ml/min.

hemoglobin and glycated albumin, and AGEs, such as N^ϵ -(carboxymethyl)lysine and N^ϵ -(carboxyethyl)lysine, in blood and a range of tissues (15–18). Protein glycation has been implicated in the development of diabetes and thereby contributes to the physical disability and high mortality rate found in diabetic patients (19, 20).

Although protein glycation has been investigated thoroughly, little attention has been paid to lipid glycation. Because of the presence of a free amino group, it seems logical to assume that aminophospholipid can be modified by glycation (21). *In vivo* lipid glycation is likely to induce changes in the biosynthesis and turnover of membrane phospholipids, the physical properties of membrane, the activities of membrane-bound enzymes, and the susceptibility of oxidative stress. These changes may contribute to the pathology of aging and chronic disease, such as diabetes and atherosclerosis. Although the importance of lipid glycation to human physiology is increasing, a lipid glycation inhibitor has not been discovered. To discover an effective inhibitor, a screening system is essential. Therefore, the objectives of this study were to optimize the reaction conditions between PE and glucose and to develop a lipid glycation model useful for inhibitor screening.

Initially, we investigated the optimal reaction conditions (glucose concentration, temperature, buffer type, and pH) for dioleoyl-PE glycation (Fig. 3). High glucose concentration (300–500 mM) stimulated Schiff-PE formation, whereas temperature did not. Similar results were reported by Ravandi et al. (22). Therefore, lipid glycation

kinetics would depend on glucose concentration, especially the open-chain form of glucose (Fig. 1). Methanolic buffer (70–80% methanol) enhanced Schiff-PE formation. Because excess methanol (>80%) in buffer causes instability of pH, we decided to use 70% methanolic buffer. According to buffer type and pH, phosphate buffer (pH 7.0–7.4) was the most effective for Schiff-PE formation. The accelerating effect of phosphate anion on the Maillard reaction is well known (23–25). It is likely that phosphate anion could enhance lipid glycation by a neighboring catalyst effect.

Once the conditions described above were fully optimized, the nonenzymatic conversion of dioleoyl-PE to Amadori-PE via Schiff base was seen (Fig. 4). Interestingly, rearrangement of Schiff-PE to Amadori-PE began at 12 h after incubation. In contrast, protein glycation (Amadori formation) generally occurs after incubation for several weeks (16). Therefore, the kinetics of lipid glycation is faster than that of protein glycation.

Next, using our lipid glycation system, we investigated compounds bearing antiglycative properties. Protein glycation inhibitors (aminoguanidine, pyridoxamine, and carnosine) (26–30), antioxidants (ascorbic acid, α -tocopherol, quercetin, and rutin), and other compounds (L-lysine, L-cysteine, pyridoxine, pyridoxal, and pyridoxal 5'-phosphate) were evaluated for their antiglycative properties. Unexpectedly, some components regarded as protein glycation inhibitors and antioxidants had little inhibitory effect on lipid glycation (Fig. 5). The most active

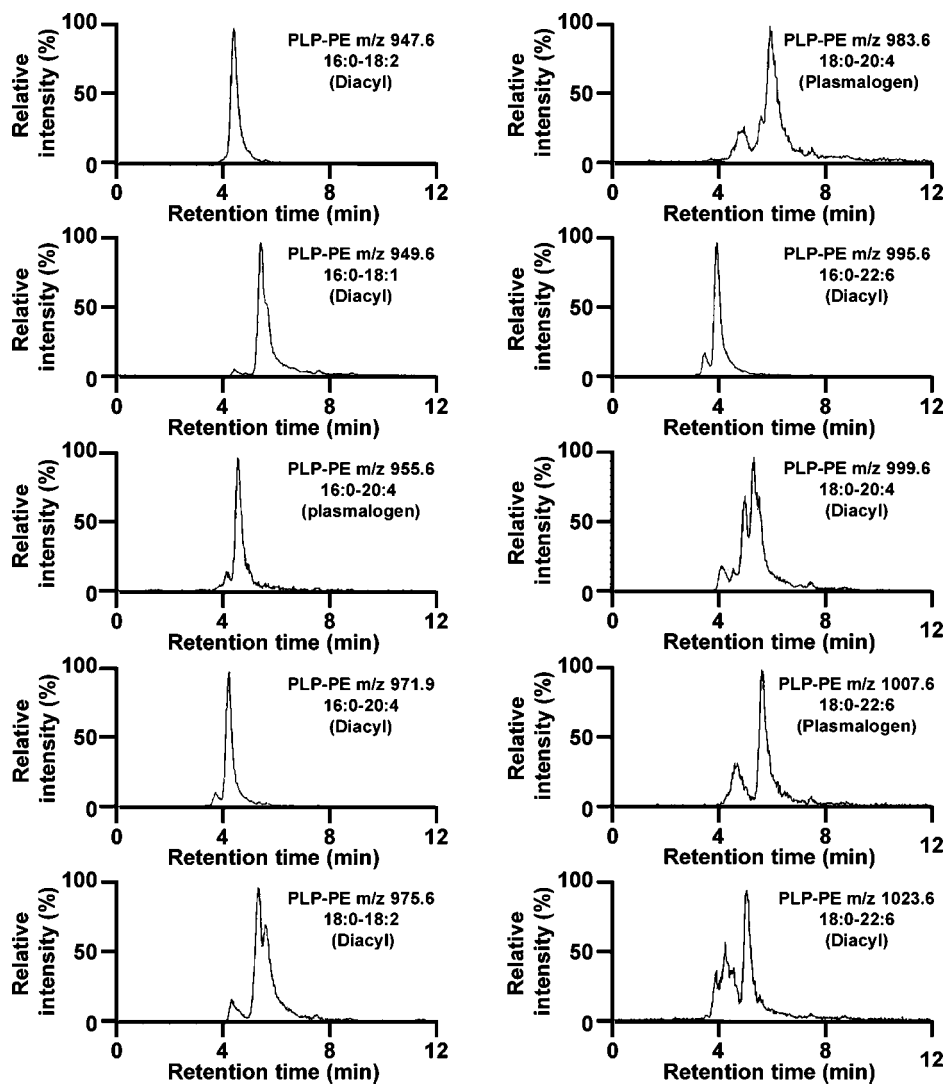


Fig. 11. Typical multiple reaction monitoring (MRM) chromatograms of PE-pyridoxal 5'-phosphate adduct (reduced form) from human RBC extract. Total lipids were extracted from human RBCs (packed cells) by the method of Rose and Oklander (11). RBC lipid extract was treated with NaBH_4 and subjected to QTRAP LC/MS/MS with MRM.

inhibitors were pyridoxal 5'-phosphate and pyridoxal (vitamin B₆ derivatives) (Figs. 5, 6). The inhibitory mechanism involved condensation of the aldehyde group of pyridoxals with the amino group of PE (Figs. 7, 8). This condensation was faster than glycation between PE and glucose. In this manner, pyridoxals showed strong inhibition of lipid glycation.

Vitamin B₆ refers to a collective of six biologically interconvertible 3-hydroxy-2-methylpyridine compounds: pyridoxal, pyridoxine, pyridoxamine, and their respective 5'-phosphates. Of these, pyridoxal 5'-phosphate is the active form of vitamin B₆, serving as a coenzyme in numerous processes, including tryptophan-niacin conversion, heme synthesis, gluconeogenesis, neurotransmitter synthesis, and amino acid metabolism (31, 32). In addition, pyridoxal 5'-phosphate acts as a coenzyme in the catabolism of homocysteine to cystathionine and

ultimately cysteine (32). As a result, vitamin B₆ played a role in decreasing homocysteine, which contributes to atherosclerosis and coronary heart disease prevention (33, 34). Apart from its role as a coenzyme, recent studies suggested that vitamin B₆ can act as a chemopreventive agent. It was reported that pyridoxal 5'-phosphate suppresses tumor growth (35) and that pyridoxamine prevents protein glycation (AGE formation) (28). Thus, the biological role and chemopreventive effect of vitamin B₆ have attracted considerable attention. In this study, PE-pyridoxal 5'-phosphate adduct was detectable in human RBCs (Figs. 9–11), suggesting a new role of vitamin B₆ as a lipid glycation inhibitor. Accordingly, we conducted an animal study and found that increased plasma Amadori-PE and PCOOH concentrations in STZ-induced diabetic rats could be decreased by dietary supplementation of pyridoxal 5'-phosphate (Fig. 12). Furthermore, dietary

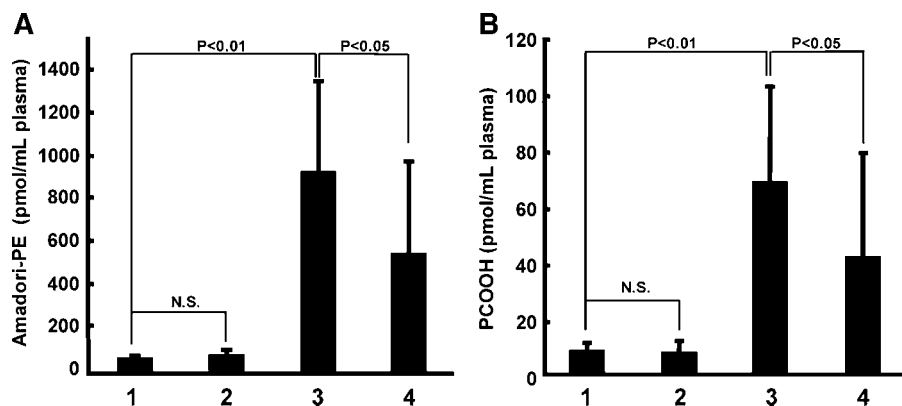


Fig. 12. Effect of dietary pyridoxal 5'-phosphate on plasma Amadori-PE (A) and phosphatidylcholine hydroperoxide (PCOOH) (B) in streptozotocin (STZ)-induced diabetic rats. Bar 1, normal rats (control); bar 2, normal rats receiving 2 mM aqueous pyridoxal 5'-phosphate (equivalent to 300 mg/kg body weight/day); bar 3, diabetic STZ rats; bar 4, diabetic rats receiving 2 mM aqueous pyridoxal 5'-phosphate. Plasma Amadori-PE (18:0-22:6-Amdori-PE) concentration was determined by QTRAP LC-MS/MS (5, 6). Plasma PCOOH was quantified by chemiluminescence detection-LC (2). Values are means \pm SD (n = 6).

pyridoxal 5'-phosphate tended to improve the lipid metabolism (i.e., the abnormal increase of neutral lipids in plasma) of STZ rats (Fig. 13). Therefore, it is likely that dietary pyridoxal 5'-phosphate can inhibit both lipid glycation and lipid peroxidation *in vivo*, which might contribute to the prevention of diabetes. Nevertheless, more studies are required to further elucidate the effects of lipid glycation inhibitors on lipid peroxidation (i.e., PCOOH formation), oxidative stress, the pathophysiology of human disease, and food deterioration.

In the field of protein glycation, it has been recognized that the discovery or design of a protein glycation inhibitor could provide a therapeutic approach for diabetes prevention (26). For example, amino compounds (e.g., alanine) can react with glucose and minimize the potentially deleterious consequences of hyperglycemia (36).

Hydrazine compounds trap reactive carbonyls formed during the Maillard reaction, especially Amadori intermediates, thus preventing their conversion to AGEs (36). Similarly, aminoguanidine is well known as an AGE inhibitor (27). We believe that our screening system could lead to the discovery of a new type of glycation inhibitor (i.e., pyridoxal 5'-phosphate) for the purpose of lipid glycation prevention.

In conclusion, although recent studies have suggested a relationship between lipid glycation and diseases (5–9, 21, 37–40), a lipid glycation inhibitor has not been discovered. In this study, using our lipid glycation system, we found that pyridoxal 5'-phosphate and pyridoxal exhibited significant antiglycative effects. Hence, these pyridoxals have potential use as therapeutic compounds for diabetes prevention. **■**

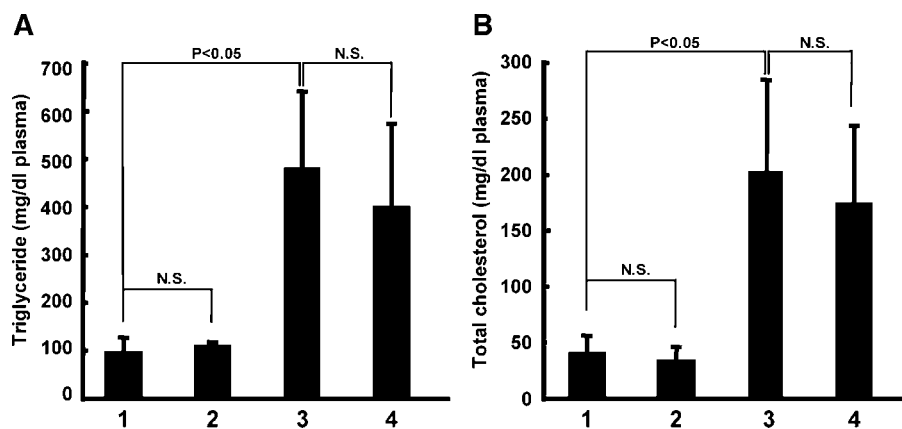


Fig. 13. Effect of dietary pyridoxal 5'-phosphate on plasma triglyceride and cholesterol in STZ-induced diabetic rats. Bar 1, normal rats (control); bar 2, normal rats receiving 2 mM aqueous pyridoxal 5'-phosphate (equivalent to 300 mg/kg body weight/day); bar 3, diabetic STZ rats; bar 4, diabetic rats receiving 2 mM aqueous pyridoxal 5'-phosphate. Plasma cholesterol and triglyceride were measured enzymatically with commercial kits. Values are means \pm SD (n = 6).

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