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Synthetically prepared Amadori-glycated phosphatidylethanolamine can trigger lipid peroxidation via free radical reactions

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Abstract This study for the first time confirmed the peroxidative role of the Amadori product derived from the glycation of phosphatidylethanolamine (PE), namely Amadori–PE. The product was synthesized from the reaction of dioleoyl PE with D-glucose, and then purified by a solid-phase extraction procedure, which was a key step in the next HPLC technique for the isolation of essentially pure Amadori–PE. When the synthetically prepared Amadori–PE was incubated with linoleic acid in the presence of Fe^{3+} in micellar system, a remarkable formation of thiobarbituric acid reactive substances was observed together with increases in lipid hydroperoxides. In addition, the lipid peroxidation caused by Amadori–PE was effectively inhibited by superoxide dismutase, mannitol, catalase and metal chelator. These results indicated that Amadori–PE triggers oxidative modification of lipids via the generation of superoxide, and implied the involvement of 'lipid glycation' along with membrane lipid peroxidation in the pathogenesis of diabetes and aging.

Key words: Glycation; Phosphatidylethanolamine; Amadori product; Lipid peroxidation; Solid-phase extraction; Diabetes

1. Introduction

The oxidative modification of lipids in vivo has been recognized to play a central role in atherogenesis and to contribute to the diverse vascular sequelae of diabetes and aging [1,2]. However, despite extensive investigation into the biological role of lipid peroxidation, there has been little insight into the biochemical processes that initiate lipid peroxidation in vivo. In vitro studies on the Maillard reaction [3] have demonstrated that protein-bound Amadori products bear reactive oxygen species. The reactive oxygen species are apt to cause the peroxidation of unsaturated fatty acid residues, which propagate free radical reactions. Since the Maillard reaction, occasionally referred to as a glycation, widely occurs in biological systems, the reaction could play an important and perhaps primary role in initiating lipid peroxidation in vivo.

In 1993, Bucala et al. [4] first proposed that the free amino groups of aminophospholipids would be targets for the glycation, which expanded the concept of the Maillard reaction in biological systems. Subsequent in vitro studies [5–7] have shown that phosphatidylethanolamine (PE) reacts with glucose, leading through an unstable Schiff base to a PE-linked Amadori product (Amadori–PE; Scheme 1). Hence, it seemed logical to anticipate that the Amadori–PE may generate reactive oxygen species, and thus induce lipid peroxidation, as has already been demonstrated in the case of protein-bound Amadori compounds [3]. These reactions are likely to be steps in the presumable pathways for generating oxidative stress in vivo. Excessive glycation of membrane lipids could conceivably cause membrane lipid peroxidation, which results in a deteriorated membrane structure. Such abnormalities might lead to disorder in the maintenance of cellular integrity and survival contributing to the pathogenesis. However, so far, the role of Amadori–PE as well as other glycated lipids in lipid peroxidation has never been ascertained, mainly because of the difficulty in preparing authentic glycated lipids with the available methods. Here, we describe the development of high performance liquid chromatography (HPLC) coupled with a solid-phase extraction method for a rapid and reliable preparation of Amadori–PE. We initially attempted to use the solid-phase extraction procedure using a Bond Elut phenylboronic acid (PBA) cartridge; this extraction was a key step for obtaining Amadori–PE as an essentially pure compound. Finally, using the synthetically prepared Amadori–PE, we demonstrated, for the first time, that the Amadori–PE induces remarkable lipid peroxidation.

2. Materials and methods

2.1. Materials

1,2-Di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (dioleoyl PE) was purchased from Funakoshi (Tokyo, Japan). D-glucose, linoleic acid, mannitol, and superoxide dismutase (SOD) (Mn type, EC 1.15.1.1) from *Bacillus* sp. were obtained from Wako (Osaka, Japan). Catalase (C-10, EC 1.11.1.6) from bovine liver was from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Glycation of PE

Dioleoyl PE (27 μmol) was incubated with D-glucose (2.0 mmol) in 30 ml of 0.1 M phosphate buffer/methanol (2:1, v/v, pH 7.4) at 37°C according to our previous report [7]. At different time intervals (0–20 days), an aliquot of the incubation mixture was collected, and was then mixed with chloroform/methanol (2:1, v/v) to extract lipid-soluble products [7]. These extracts were subjected to HPLC with on-line mass spectrometry (HPLC-MS) for monitoring the yield of Amadori–PE formation. HPLC-MS analysis was performed using a Mariner electrospray ionization time of flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) with a NANOSPACE Liquid Chromatograph (Shiseido, Tokyo, Japan). An ODS column (TSKgel ODS-80Ts, 4.6×250 mm; Tosoh, Tokyo, Japan) was used with a mixture of methanol/water (99:1; containing 5 mM ammonium acetate) at a flow rate of 1 ml/min. Positive ionization spectra were taken in the *m/z* range 200–1200.

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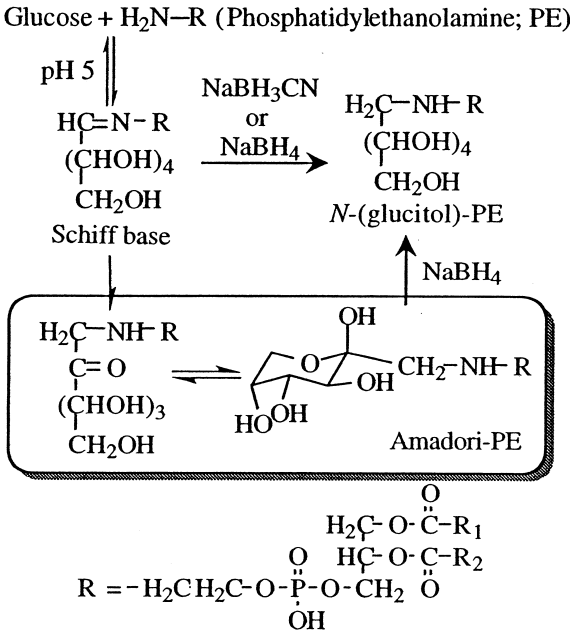


Scheme 1. Scheme for the glycation of PE. Glucose reacts with the amino group of PE to form an unstable Schiff base, which undergoes an Amadori rearrangement to yield the stable deoxy-D-fructosyl PE, namely Amadori–PE. The Schiff base is readily discharged by treatment with mild acid (pH 5), but Amadori–PE is acid-stable. Also, the Schiff base is reducible by either $NaBH_3CN$ or $NaBH_4$ to the *N*-(glucitol) derivative of PE, while the Amadori product is reducible by $NaBH_4$ only [17].

2.3. Purification and identification of Amadori–PE

Amadori–PE was purified by HPLC after solid-phase extraction using a Varian Bond Elut PBA cartridge (Harbor City, CA, USA), according to the following procedures. The resultant lipid extract was redissolved in 1 ml of methanol/30% ammonium hydroxide (95:5, v/v), and then loaded onto a PBA cartridge equilibrated in the same solvent. The cartridge was rinsed with an additional 3 ml of methanol/30% ammonium hydroxide (95:5, v/v) and the eluent was discarded. Amadori–PE was recovered with 5 ml of methanol, and finally purified by HPLC using the same conditions as described above. The accurate mass for Amadori–PE was determined by JEOL-JMS-700 mass station (JEOL, Tokyo, Japan) in high resolution fast atom bombardment mode (HRFAB). HRFAB-MS measurement was performed in the positive mode. To assess the structure of Amadori–PE, the substance (100 nmol) was incubated in 1 ml of 0.1 M sodium acetate buffer (pH 5.0) for 60 min at 37°C, or in 1 ml of methanol containing either 50 mM $NaBH_3CN$ or 50 mM $NaBH_4$ for 60 min at room temperature. Before and after these incubations, samples were extracted with chloroform/methanol (2:1, v/v), and then analyzed by HPLC-MS.

2.4. In vitro incubation experiments

To investigate the effect of Amadori–PE on lipid peroxidation, micellar system was employed. Briefly, linoleic acid (5.2 mM) was dispersed in 5 ml of 50 mM sodium dodecylsulfate (SDS) aqueous solution [8], and this linoleic acid micellar solution was incubated alone or with either Amadori–PE (3, 6 or 12 μM) or dioleoyl PE (6 μM) in the presence or absence of 30 μM metal ion such as Fe^{3+} and Fe^{2+} at 37°C. In the experiment on the effects of reactive oxygen inhibitors, either SOD (50 or 500 units/ml), mannitol (50 or 200 mM), catalase (100 or 500 units/ml) or EDTA (5 mM) was added to the incubation mixture. At different time intervals (0–60 min), lipid peroxidation was assessed by quantifying the formation of thiobarbituric acid reactive substances (TBARS) and of lipid hydroperoxides. TBARS were measured by the colorimetric method of Ohkawa et al. [9] with 0.01% butylated hydroxytoluene in the assay mixture. Lipid hydroperoxides were quantified by the FOX assay [10].

2.5. Statistical analysis

The data were expressed as the mean and standard deviations (S.D.). Statistical comparisons were made with Student's *t*-test.

3. Results

Fig. 1A shows the typical total ion current (TIC) chromatogram of the resultant lipid extract from the reaction mixture of dioleoyl PE and D-glucose. A number of small peaks along with the unreacted PE were detected in the extract. Among these peaks, a peak component at retention time of 11.3 min gave molecular ion $[M+H]^+$ at *m/z* 906.6 identical to Amadori–PE (Fig. 1B). However, this peak overlapped in part with proximal peak components, and was too small to isolate as a pure compound under the present HPLC conditions (Fig. 1A). Thus, to prepare the fraction enriched with Amadori–PE, the resultant lipid extract was applied to a solid-phase extraction prior to HPLC analysis. As shown in Fig. 1C, this procedure was a very useful step in the next HPLC technique for the isolation of Amadori–PE. Typical TIC and single ion chromatograms of the isolated Amadori–PE are shown in Fig. 2. As judged from these chromatograms, Amadori–PE was an essentially pure compound, comprised of neither dioleoyl PE nor other compounds. The accuracy of the isolated

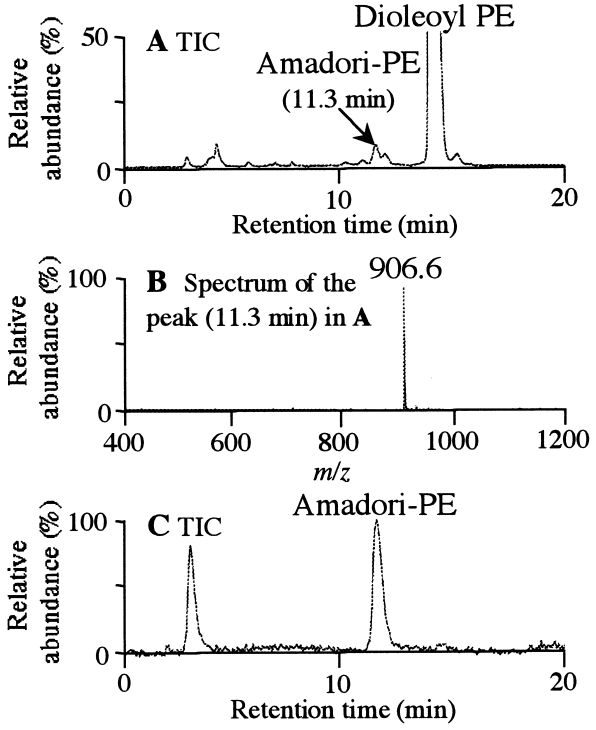


Fig. 1. Production of Amadori–PE during glycation and its purification. A: Total ion current (TIC) chromatogram as obtained by LC-MS for the lipid extract from a 15-day incubation mixture of dioleoyl PE and D-glucose. B: Mass spectrum of the peak (11.3 min) detected in the chromatogram A. On the other hand, the same lipid extract was purified by solid-phase extraction and then submitted to LC-MS (C). Dioleoyl PE (27 μmol) was incubated with D-glucose (2.0 mmol) in 30 ml of 0.1 M phosphate buffer/methanol (2:1, v/v, pH 7.4) at 37°C up to 20 days. Amadori–PE formation reached a maximum after a 15-day incubation (data not shown); the sample was used for the preparation of standard Amadori–PE. Fractions with retention time of 10.8–11.8 min in the chromatogram C were collected and dried, yielding the essentially pure Amadori–PE.

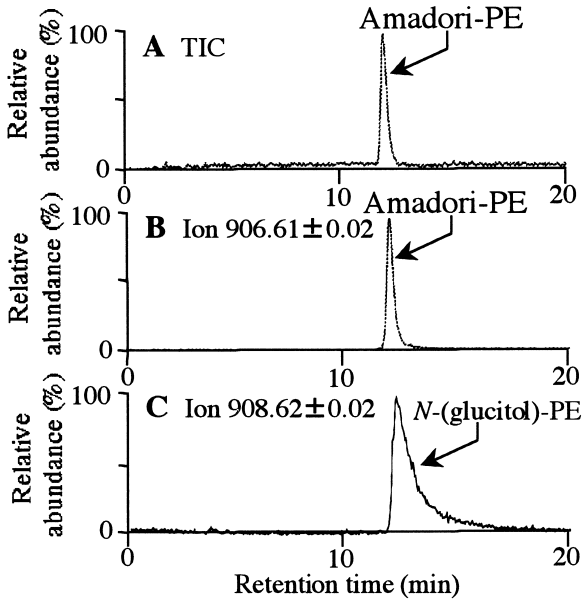


Fig. 2. LC-MS analysis of the synthetic Amadori–PE. The upper chromatogram (A) represents the total ion current (TIC); the lower chromatogram (B) shows single ion plot of the mass corresponding to the $[M+H]^+$ ion of Amadori–PE (*m/z* 906.61). Amadori–PE (1.0 nmol) was analyzed by LC-MS. On the other hand, following reduction of Amadori–PE by $NaBH_4$, the occurrence of *N*-(glucitol)-PE ($[M+H]^+$ 908.62) was detected (C). The accurate mass of *N*-(glucitol)-PE was determined as *m/z* 908.6233 $[M+H]^+$ (908.6228, calculated for $C_{47}H_{91}N_1O_{13}P_1$).

Amadori–PE was confirmed by HRFAB-MS analysis; *m/z* 906.6064 $[M+H]^+$ (906.6072, calculated for $C_{47}H_{89}N_1O_{13}P_1$).

In general, Schiff base and Amadori compound are formed during the initial stage of the Maillard reaction. In the present study, a small amount of PE-linked Schiff base as well as Amadori–PE were produced during dioleoyl PE glycation (data not shown). Since these compounds show the same mass, special care is needed to distinguish them. As shown in Scheme 1, Schiff base is readily discharged by treatment with mild acid and is reducible by either $NaBH_3CN$ or $NaBH_4$. On the contrary, Amadori product is acid-stable and is reducible by $NaBH_4$ only. In this study, the isolated Amadori–PE behaved exactly according to the general rules of Amadori compound (Table 1 and Fig. 2C). Therefore, we concluded that the substance is deoxy-D-fructosyl PE as we previously estimated [7], that is an Amadori product formed by the amino-carbonyl reaction of dioleoyl PE with D-glucose.

Using the synthetically prepared Amadori–PE, we investigated its role in initiating lipid peroxidation in the presence or absence of transition metal ion in micellar system. When Amadori–PE (3–12 μM) was incubated with linoleic acid in the presence of Fe^{3+}, the system clearly showed lipid peroxidation which was monitored by the formation of TBARS (Fig. 3A). The lipid peroxidation increased almost linearly over 60 min, and was enhanced in proportion to the Amadori–PE concentration (Fig. 3A). To further characterize the peroxidative action of Amadori–PE, linoleic acid was incubated alone or with either 6 μM Amadori–PE or 6 μM dioleoyl PE (non-glycated PE) for 60 min. As shown in Fig. 3B, incubation of a mixture of linoleic acid and Fe^{3+} showed TBARS at a concentration of 6.7 μM. This value was significantly increased to 10.2 μM by the addition of Amadori–PE to the incubation mixture, whereas the value did not change (6.4 μM) by the addition of dioleoyl PE. Similar results were confirmed in the formation of lipid hydroperoxides (41 μM for linoleic acid/Fe^{3+} system; 65 μM for Amadori–PE/linoleic acid/Fe^{3+} system; 37 μM for dioleoyl PE/linoleic acid/Fe^{3+} system). On the other hand, the TBARS production caused by Amadori–PE was somewhat reduced using Fe^{2+} instead of Fe^{3+}, and was not confirmed in subsequent experiments performed in the absence of metal ion (Fig. 3B). When Amadori–PE (6 μM) was incubated alone in SDS solution for 60 min, TBARS production was not confirmed (data not shown). As shown in Fig. 4, the TBARS formation in the Amadori–PE/

Table 1
Structural stability of Amadori–PE after treatment with mild acid or reducing agents[a]

Conditions	Remaining Amadori–PE (% of control)
Before treatment (control)	100
After mild acid (pH 5.0) treatment	98 ± 13
After $NaBH_3CN$ reduction	91 ± 14
After $NaBH_4$ reduction	28 ± 10[b]

[a]Incubation conditions are as given in Section 2. Values were expressed as percentage of the amount of Amadori–PE before reaction. Means ± S.D. ($n = 3$).
[b]Following 60 min reduction, the occurrence of *N*-(glucitol)-PE as a reduced product of Amadori–PE was confirmed by LC-MS (details in Fig. 2C). When Amadori–PE was treated with $NaBH_4$ for 24 h, it was completely reduced.

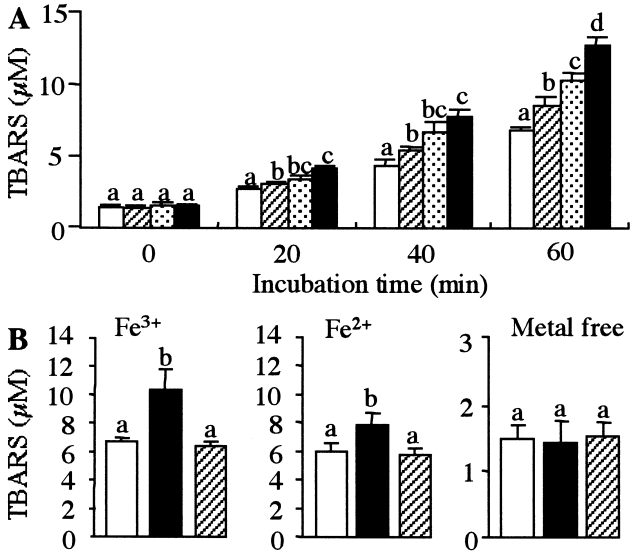


Fig. 3. Lipid peroxidation induced by Amadori–PE. A: linoleic acid (5.2 mM) was dispersed in 5 ml of 50 mM SDS solution, and this linoleic acid micellar solution was incubated alone or with 3–12 μM Amadori–PE in the presence of 30 μM Fe^{3+} at 37°C. At each time point TBARS was assayed. White bars, linoleic acid only; hatched bars, linoleic acid+3 μM Amadori–PE; stippled bars, linoleic acid+6 μM Amadori–PE; black bars, linoleic acid+12 μM Amadori–PE. B: The SDS-dispersed linoleic acid (5.2 mM) was incubated alone or either with Amadori–PE (6 μM) or with dioleoyl PE (6 μM) in the presence or absence of metal ion (Fe^{3+} or Fe^{2+}; 30 μM) for 60 min. White bars, linoleic acid only; black bars, linoleic acid+ Amadori–PE; hatched bars, linoleic acid+dioleoyl PE. Values not sharing a common superscript are significantly different at $P < 0.05$. Values are means ± S.D. ($n = 3$).

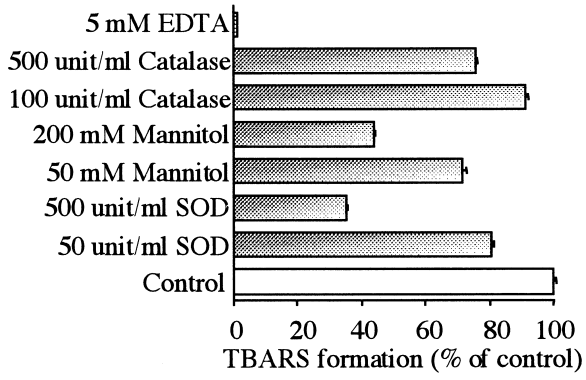


Fig. 4. Effect of reactive oxygen inhibitors on the Amadori–PE-induced lipid peroxidation. Inhibitors were incubated in SDS micellar solution containing Amadori–PE (6 μM), linoleic acid (5.2 mM) and 30 μM Fe^{3+} at 37°C for 60 min. After incubations, TBARS was assayed. Values are means ± S.D. ($n = 3$).

linoleic acid/Fe^{3+} system was effectively inhibited by SOD, mannitol, catalase and EDTA.

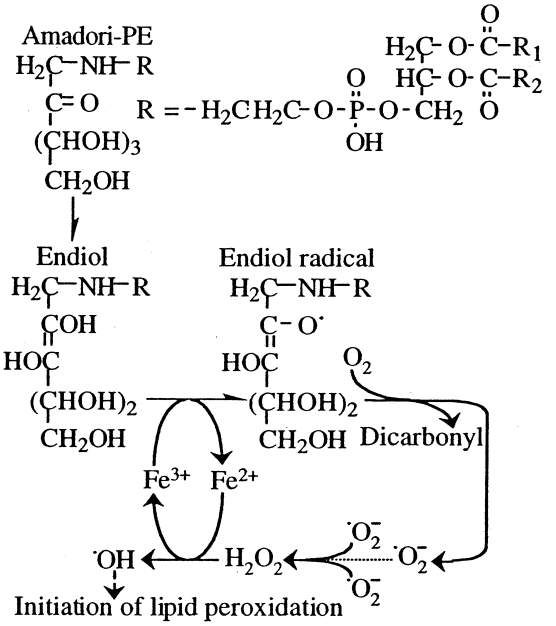
4. Discussion

In the present study, Amadori compound from glycation of PE was prepared as an essentially pure form and its role in initiating lipid peroxidation was assessed. When Amadori–PE was incubated with linoleic acid in the presence of metal ion, a significant amount of TBARS was detected, which was quenched by the addition of reactive oxygen inhibitors. These findings indicated for the first time that Amadori–PE is capable of generating reactive oxygen species, which trigger lipid peroxidation.

To date, there have been only two reports on the preparation method of Amadori–PE [7,11]. We first isolated Amadori–PE from the reaction mixture of egg yolk PE and D-glucose by employing HPLC [7]. However, the Amadori–PE isolated previously [7] may have been contaminated, because the UV spectrum of isolated compound showed an unassigned absorbance at 291 nm. Most recently, Utzmann and Lederer [11] reported a synthetic method for Amadori–PE, although their method requires a series of reactions leading to its synthesis. Hence, in this study, we attempted to develop a simple and more effective method for the preparation of Amadori–PE. The production of Amadori–PE during glycation was confirmed by HPLC-MS analysis (Fig. 1A and B), but we were unable to isolate the Amadori–PE by the HPLC technique alone. Therefore, we employed the solid-phase extraction using a phenylboronic acid (PBA) cartridge prior to HPLC analysis. In general, the *cis*-diol structure found in various biomolecules potentially forms the complex with boronic acid under alkaline conditions [12]. This '*cis*-diol-boronic acid' complex can easily dissociate under neutral or acidic conditions. Thus, the presence of *cis*-diol configuration in Amadori–PE would be available for the purification. In this study, the resultant lipid extract was loaded to a PBA cartridge, and then rinsed with the alkaline methanol. During these procedures, Amadori–PE was retained on the cartridge, whereas most of the impurities such as unreacted PE were eluted. Subsequently, Amadori–PE could be recovered from the cartridge with methanol, and finally purified by HPLC (Fig. 1C). The isolated substance was an essentially pure Amadori compound (Fig. 2 and Table 1). Our method for preparation of standard Amadori–PE was rapid and reliable.

Recent papers have hypothesized that aminophospholipid glycation may occur and play an important role in lipid peroxidation in vivo [4–7,13–19]. In this study, to clarify the direct effect of Amadori–PE on unsaturated fatty acid, we chose the simple reaction system; that is, Amadori–PE was incubated with linoleic acid in micellar system. As a result, we demonstrated that Amadori–PE triggers lipid peroxidation in the presence of transition metal ion during the incubation (Fig. 3). It is important to note that the amount of Amadori–PE (3–12 μM) added to the incubation mixture was rather low, and the percentage of Amadori–PE to linoleic acid was only 0.06–0.23 mol%. Since Ravandi et al. [14] have estimated that 2.3% of total PE of human plasma exists in the glycated form, it seems likely that Amadori–PE initiates peroxidation in vivo. Based on present findings (Figs. 3 and 4), a possible mechanism of Amadori–PE-induced lipid peroxidation was speculated as shown in Scheme 2. Amadori–PE generates superoxide via autoxidation that is caused by one-electron transfer in the presence of Fe^{3+}. Superoxide then dismutates to hydrogen peroxide. Further reaction of hydrogen peroxide with Fe^{2+} by the Fenton reaction gives hydroxyl radicals, which initiate lipid peroxidation. During these reactions, SOD, mannitol and catalase can inhibit the lipid peroxidation as a superoxide, a hydroxyl radical and a hydrogen peroxide scavenger, respectively (Fig. 4). In the absence of metal ion, the incubation of Amadori–PE with linoleic acid did not yield a significant production of TBARS (Fig. 3B). This would indicate that Fe^{3+} is needed for the Amadori–PE-induced lipid peroxidation. On the other hand, commercially available linoleic acid containing its hydroperoxide (0.3 mol%) was used in the present experiments. Amadori–PE-induced lipid peroxidation was also confirmed by using linoleic acid free from peroxide, which was prepared according to the method of Fukuzawa et al. [8].

Recently, Ravandi et al. [16,18,19] have reported that the



Scheme 2. Proposed mechanism for the Amadori–PE-mediated lipid peroxidation.

oxidation of low density lipoprotein (LDL) enriched with synthetically prepared glycated PE resulted in a significant increase in the accumulation of LDL lipid oxidation products when compared with the oxidation of LDL enriched in non-glycated PE. They [16,18] proposed that glycated PE distorts the packing of the lipid layer and thereby renders membrane lipids more susceptible to oxidation. Ravandi et al. [5,14,16,18,19] expounded the glycated PE used in their early studies as Schiff base, but used in recent papers as Amadori product. Explicit structural proofs and purity of glycated PE were not given in their papers.

Under hyperglycemic conditions (i.e. diabetes), many proteins in blood and tissue organelles are exposed to glycation. In the same way, aminophospholipid glycation might occur in vivo. In diabetic plasma, an increase of metal ions such as iron [20] and copper [21] was found. Under such situations, Amadori–PE is apt to induce notable lipid peroxidation. On the other hand, once Amadori–PE is formed, it may undergo a series of complex reactions to produce the advanced glycation end products (AGEs) such as carboxymethyl– and pyrrolecarbaldehyde–PE [11,15]. We are now attempting to quantify Amadori–PE as well as AGEs in human plasma and red blood cells to prove the involvement of lipid glycation in the pathogenesis of diabetes and aging.

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