Isolation and identification of glycated aminophospholipids from red cells and plasma of diabetic blood

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Abstract Glycosylation is a major pathway for posttranslational modification of tissue protein and begins with nonenzymatic addition of carbohydrate to the primary amino groups. Excessive glycation of tissue protein has been implicated in the pathogenesis of diabetes and ageing. While glycation of aminophospholipids has also been postulated, glycated aminophospholipids have not been isolated. Using normal phase HPLC with on-line electrospray mass spectrometry we found glycated ethanolamine phospholipids to make up 10–16% of the total phosphatidylethanolamine (PE) of the red blood cells and plasma of the diabetic subjects. The corresponding values for glycated PE of control subjects were 1–2%.

Key words: Glucosylated aminophospholipid; Glucose; Phosphatidylethanolamine; Phosphatidylserine; Electrospray; Thin-layer chromatography; Liquid chromatography, mass spectrometry; Normal phase HPLC

1. Introduction

We have previously reported on the identification of high molecular weight secondary products of peroxidation of standard lipids and lipoproteins during in vitro incubation with *tert*-butyl hydroperoxide [1,2] or copper ions [3,4]. Using these products as reference standards we have identified lipid ester hydroperoxides and core aldehydes in plasma and atheromas of patients with atherosclerosis and diabetes [5]. Bucala et al. [6] have postulated that aminophospholipids might react with the increased glucose in diabetes to form Schiff bases, which promote fatty acid oxidation. The glycation products of amino group-containing phospholipids, however, have never been isolated and their role in lipid peroxidation has not been directly determined. We have recently synthesized glucosylated phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS) and have determined their chromatographic properties [7]. In the present study we have used the glucosylated aminophospholipids as reference compounds to identify and quantitate glucosylated PE in lipid extracts from red blood cells and plasma of diabetics and of control subjects.

2. Materials and methods

Glucosylation products of the dipalmitoyl species of PE and PS as well as the PE of egg yolk and PS of human red blood cells were available from previous work [7]. Non-glucosylated egg yolk PE and bovine brain PS, as well as egg yolk phosphatidylcholine (PC) and bovine brain phosphatidylinositol (PI) and sphingomyelin (SPH) were obtained from Sigma Chemical Co., St. Louis, MO. All chemicals were of reagent grade quality, while the solvents were of chromatographic purity and were obtained from local suppliers. The purity of the reference compounds was ascertained by thin-layer chromatography (TLC) [3,7].

2.1. Isolation of phospholipids from blood

Blood was obtained from six diabetic patients and six non-diabetic donors. The diabetics were selected for elevated blood glucose levels indicated by their content of glycosylated hemoglobin (9–15%). EDTA blood was centrifuged (2300×*g* for 10 min) in a swinging bucket rotor to separate the plasma from the red cells. The cells were washed three times with five volumes of phosphate buffered saline (150 mM NaCl, 50 mM sodium phosphate, pH 8.0) and centrifuged (2300×*g* for 10 min). The red blood cell phospholipids were extracted according to Rose and Oaklander [8]. The plasma phospholipids were extracted with chloroform-methanol 2:1 modified from Folch et al. [9]. Glucosylated PE could be stored at −20°C in neutral chloroform–methanol for several days without decomposition. The Schiff base dissociated in dilute acetic acid.

2.2. $NaCNBH_4$ reduction

Aliquots of phospholipids containing glucosylated PE in chloroform were reduced by adding freshly prepared $NaCNBH_4$ in methanol (final concentration 70 mM) as previously described [7].

2.3. Normal phase HPLC LC/ESI/MS of phospholipids

Normal phase HPLC separations of phospholipids were performed on Spherisorb 3 micron columns (100 mm×4.6 mm ID, Analtech, Deerfield, IL) installed into a Hewlett-Packard (Palo Alto, CA) Model 1090 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B Quadrupole mass spectrometer equipped with a nebulizer assisted electrospray interface. The column was eluted with a linear gradient of 100% Solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5:0.5, by vol) in 14 min, then at 100% B for 10 min [10]. Both negative and positive ionization spectra were taken in the mass range 400–1100. Selected ion spectra were retrieved from the total ion spectra by computer. The molecular species of the various glycerophospholipids were identified on the basis of the molecular mass provided by the mass spectrometer, the knowledge of the fatty acid composition of the aminophospholipid class, and the relative elution order (the less polar long chain species emerging ahead of the more polar short chain species) of the phospholipids from the normal phase column.

3. Results

Fig. 1A shows the total positive ion current profile of red blood cell phospholipids as obtained by LC/MS for a 50 year old patient with diabetes. Only PC, SPH and lysophosphatidylcholine (LPC) are seen as they are readily ionized under these conditions, but PE can also be discerned. The PC and SPH are resolved into two or more subfractions with the longer chain species being eluted earlier than the shorter chain species. LC/ESI/MS yielded molecular masses

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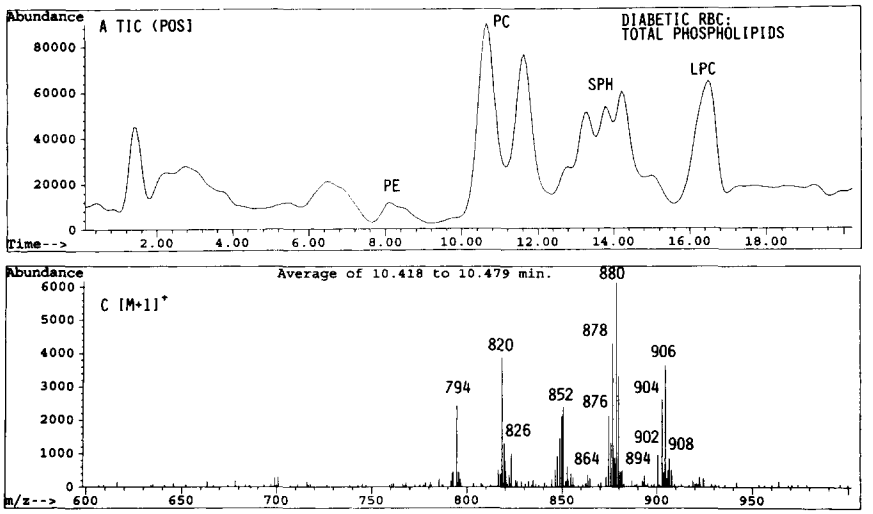

Fig. 1. LC/ES/MS of the phospholipids of red blood cells of a male diabetic subject with 9% glycation of hemoglobin. (A) Total positive ion profile; (C) mass spectra averaged over the front part of the earliest emerging PC peak (10.418–10.479 min). PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine. The masses in C correspond to the $[M+1]^+$ ions of the 18:0–18:1 (m/z 908), 18:1–18:1 (m/z 906); 18:1–18:2 (m/z 904); 16:0–20:4 (m/z 902); 16:0–18:0 (m/z 882); 16:0–18:1 (m/z 880); 16:0–18:2 (m/z 878); and 16:0–16:0 (m/z 854) of glucosylated PE. HPLC conditions: Column, Spherisorb 3 micron (100 mm×4.6 mm ID, Analtech, Deerfield, IL); Solvent: Linear gradient of 100% Solvent A to 100% Solvent B in 14 min, then Solvent B for 10 min. Solvent A: chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5, by vol). Solvent B: chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:0.5, by vol). MS instrumentation and operating conditions were as given in section 2.

characteristic of the major molecular species in each phospholipid class, except for the species associated with the front of the first PC peak, which were of higher molecular mass.

Fig. 1C gives the mass spectra averaged over the front part of the earliest emerging PC peak (10.418–10.479 min) in Fig. 1A. The major masses correspond to the $[M+1]^+$ ions of the mono- and di-unsaturated species of glucosylated PE, e.g. m/z 852 (16:0–16:1), m/z 878 (16:0–18:2), m/z 880 (16:0–18:1), m/z 882 (18:0–16:0), m/z 902 (18:2–18:2), m/z 904 (18:1–18:2), m/z 906 (18:1–18:1) and m/z 908 (18:0–18:1). We have shown previously [7] that glucosylated PE emerge with the front of the first PC peak, while glucosylated PS overlap with the LPC peak. The masses at m/z 794 and 820 were assigned to the 18:0″–20:4 and 18:0″–22:5 and other isobaric alkenylacyl PC species, respectively, to which they also correspond in HPLC retention time.

Fig. 2 gives single ion plots for the major species of glycated PE in Fig. 1C along with the proposed identities. These ions appeared just ahead of those corresponding to the major polyunsaturated species of PC, but there were some overlaps. The opportunity for overlapping of co-incidence ions from PC and glucosylated PE was eliminated by sodium cyanoborohydride reduction of the glucosylated PE. The reduction increased the mass of the $[M+1]^+$ ions by two mass units and led to a marked increase (14 min) in HPLC retention time, which resulted in the elution of the glucosylated PE species in the sphingomyelin range [7]. Similar results were obtained with the red blood cells of the other diabetic subjects.

Fig. 3A shows the total positive ion current profile of the

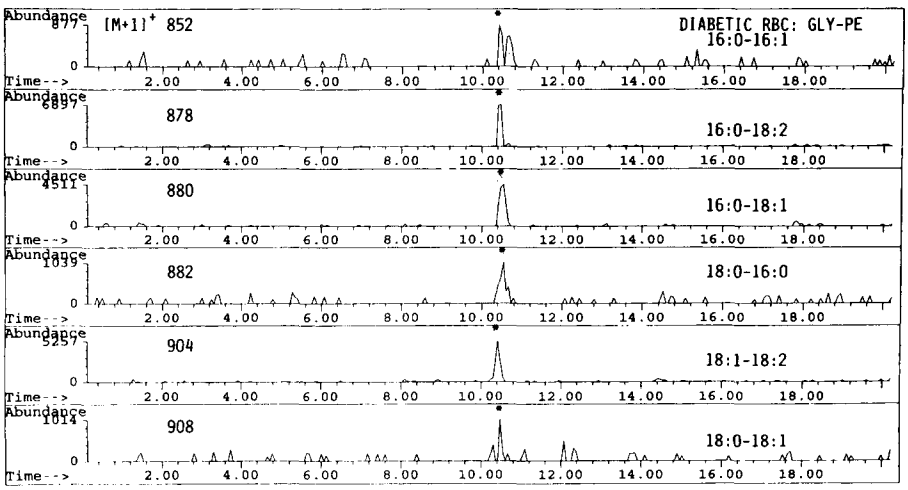

Fig. 2. Single ion plots of the masses corresponding to the $[M+1]^+$ ions of the major molecular species of glycated PE from red blood cells of a diabetic subject. The ions are identified as shown in the figure. LC/ESI/MS conditions are as given in Fig. 1.

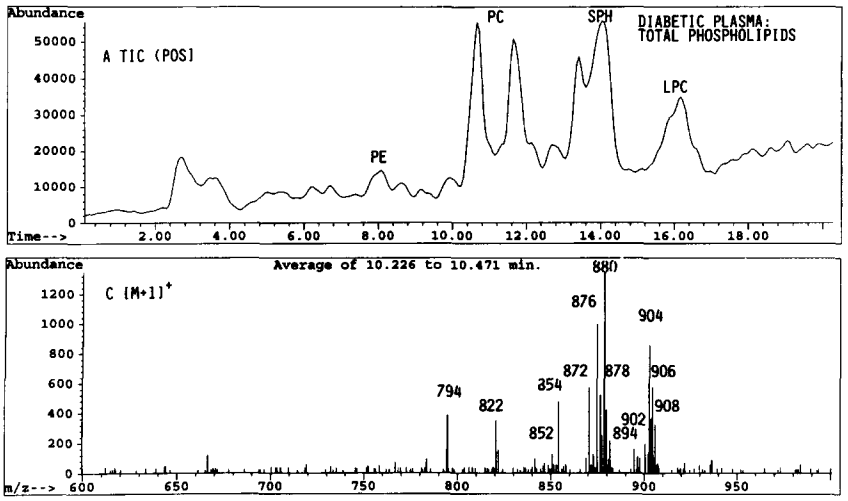

Fig. 3. LC/ES/MS of plasma phospholipids of a female diabetic with 12% glycation of hemoglobin. (A) Total positive ion current profile; (C) mass spectra averaged over the front part of the earliest emerging PC peak (10.226–10.471 min). Phospholipid classes are identified as in Fig. 1. The masses in (C) correspond to the glucosylated PE as indicated in Fig. 4. LC/ESI/MS conditions were as given in Fig. 1C.

plasma phospholipids as obtained by normal phase LC/ESI/MS for another patient with diabetes. The phospholipid classes yield a pattern slightly different from that seen for the red cells. However, PC is again split into two peaks, with the earlier emerging one overlapping with glucosylated PE. There is relatively more SPH, again being split into subfractions, and LPC in the plasma than in the red cells. Again the various phospholipid classes gave molecular masses characteristic of the major molecular species, except for the front of the first PC peak, which contained a series of higher molecular weight components corresponding to glucosylated PE.

Fig. 3C shows that the higher masses are concentrated at the front of the earliest emerging PC peak (10.226–10.471 min) in Fig. 3A. The major masses in the averaged spectrum correspond to the $[M+1]^+$ ions of glucosylated mono- and diunsaturated species of PE as noted above for the red cells, e.g. m/z 852, m/z 876, m/z 878, m/z 880, m/z 904, and m/z 908). The ions at m/z 794 and 822 correspond to the 18:0″-20:4 and 18:0″-22:4 and isobaric alkenylacyl species of PC, which are eluted earlier than the other PC species because of the lower polarity. The proportions of the glucosylated PE species differ only slightly from those of the red cells.

Fig. 4 gives single ion plots for the glucosylated PE masses in Fig. 3C along with the proposed identities of the species. Again the glucosylated species exhibited the increases in the mass and in retention time as a result of the cyanoborohydride reduction, as already noted for the glucosylated PE of the red cells. Neither positive nor negative ion mode indicated the presence of any glucosylated PS in the red cells or in plasma, although PS was demonstrated to be readily glucosylated in vitro [7].

Table 1 compares the species of the diacyl, alkenylacyl, and glycated diacyl PE from the red cells of diabetic and control subjects. Only the diacyl species have become glycated and not the alkylacyl or alkenylacyl ones, although the plasmalogenic species make up 50% of total red blood cell PE. Furthermore, the glycation of the diacyl PE appears not to be random, which contrasts with random glycation of both diacyl and plasmalogenic species in vitro [7]. It may be noted that several of the glucosylated species represent very minor red

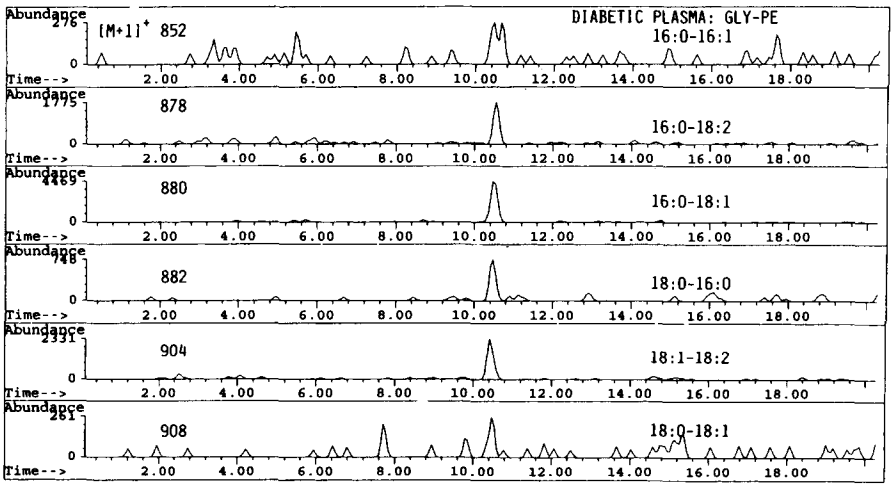

Fig. 4. Single ion plots of the masses corresponding to $[M+1]^+$ of the molecular species of glucosylated PE as obtained in Fig. 3C for the plasma of a female diabetic subject. The ions are identified as shown in the Fig. 4. LC/ESI/MS conditions are as given in Fig. 1.

Table 1
Glycated diacyl (GLY-PE), diacyl (PE) and alkenylacyl (PE″) PE in red blood cells of control and diabetic subjects (mole %)[a]

Molecular species	Controls			Diabetics		
	GLY-PE	PE	PE″	GLY-PE	PE	PE″
16:0–16:0	7.1 ± 1.1	1.2 ± 0.2		6.8 ± 0.8	2.7 ± 0.5	
16:0–18:0		1.0 ± 0.3	0.3 ± 0.1	2.5 ± 0.6	1.0 ± 0.2	
16:0–18:1		26.9 ± 5.3	5.3 ± 1.6	20.2 ± 3.7	23.6 ± 4.2	2.9 ± 0.8
16:0–18:2	70.3 ± 6.9	11.8 ± 3.2		21.7 ± 4.1	8.5 ± 2.6	0.5 ± 0.1
16:1–18:3	1.6 ± 0.5	0 .1 ± 0.1		14.2 ± 2.3	0.8 ± 0.2	
18:0–18:0		0.1 ± 0.1			0.9 ± 0.2	0.3 ± 0.1
18:0–18:1		5.1 ± 1.4	3.7 ± 1.1	1.9 ± 0.8	5.0 ± 1.8	1.7 ± 0.7
18:0–18:2	11.2 ± 2.5	8.2 ± 1.8	3.4 ± 0.9	2.7 ± 0.8	7.0 ± 1.9	2.1 ± 0.9
18:1–18:2		8.3 ± 2.3	0.9 ± 0.2	20.6 ± 4.3	9.4 ± 2.1	0.5 ± 0.1
16:0–20:4		11.7 ± 2.6	6.1 ± 1.0	3.1 ± 0.6	9.8 ± 1.8	4.6 ± 1.2
16:0–20:5	6.7 ± 1.5	0.7 ± 0.1		3.5 ± 0.7	2.1 ± 0.7	1.3 ± 0.2
16:1–20:5			2.6 ± 0.4	1.5 ± 0.3		3.0 ± 0.5
18:0–20:3			2.5 ± 0.6			0.5 ± 0.1
18:0–20:4		8.1 ± 1.8	23.5 ± 4.2		7.1 ± 1.6	18.8 ± 3.6
18:1–20:4		8.8 ± 2.1	11.0 ± 2.6		7.0 ± 1.8	8.7 ± 1.1
16.0–22.6		3.4 ± 0.8	1.1 ± 0.3		4.0 ± 1.2	6.4 ± 1.6
18.1–22.6			5.9		0.6	3.5
18.0–22.3		0.1 ± 0.1	1.1 ± 0.2		0.1 ± 0.1	0.3 ± 0.1
18.0–22.4		0.1 ± 0.1	0.7 ± 0.1			1.3 ± 0.4
18.0–22.5		0.6 ± 0.1	11.5 ± 2.1		0.7 ± 0.2	10.2 ± 2.0
18.0–22.6		1.0 ± 0.3	11.3 ± 2.3		2.4 ± 0.9	19.3 ± 3.7
18.1–22.6		1.1 ± 0.2	5.1 ± 1.8		3.9 ± 0.8	9.0 ± 1.4
Other	3.0 ± 0.8	1.9 ± 0.4	4.1 ± 1.7	1.5 ± 0.5	3.3 ± 0.8	5.1 ± 1.7
Total	1.1 ± 0.2	54.0 ± 3.5	44.9 ± 4.1	9.8 ± 1.1	40.1 ± 2.2	50.1 ± 2.9

[a]As estimated from uncorrected ion intensity by LC/ESI/MS. Means ± S.D. ($n = 6$).

cell PE components of control and diabetic subjects. The proportions of the major molecular species within the underivatized PE were comparable in the controls and diabetics, including a high proportion of the plasmalogens [11,12].

Table 2 shows that the glucosylated PE made up a somewhat higher proportion of plasma than red cell PE, although there was much less total PE in plasma. On the basis of the major PE species it was calculated that 16% and 10% of the diacyl PE of plasma and red cells, respectively, became glucosylated. The overall glycation of PE (10–16%) was similar to the overall glycation of hemoglobin (9–15%) in the diabetic patients. The control red cells and plasma showed less than 1% glucosylation.

4. Discussion

Although glucose is the least reactive of the monosaccharides in Schiff base formation [14], it has been suggested [6] that it might react with aminophospholipids and play a role in production of lipid advanced glycation end-products (AGE). In support of this hypothesis Bucala et al. [6] have reported evidence for the formation of phospholipid-linked AGE in vitro, mimicking the absorption, fluorescence and immunochemical properties of AGE that result from advanced glycosylation of proteins. Furthermore, AGE-ELISA analysis of LDL specimens isolated from diabetic individuals revealed [6] increased levels of both apoprotein and lipid-linked AGE, when compared to specimens obtained from normal, non-diabetic controls. Glycated aminophospholipids were not isolated from any of the sources.

The effect of diabetes on blood lipid composition is controversial [15]. Some studies had shown decreased [16] and others increased [17] PE levels in the red blood cells of diabetics. In one instance [17] an unidentified phospholipid had been detected by thin-layer chromatography (TLC). We estimated the Rf value of this unknown as 0.35 corresponding to an Rf value of 0.37 obtained for the standard glycated PE in the same solvent system in our laboratory. Still other studies [18] have shown significantly increased membrane lipid peroxidation and a formation of an adduct of phospholipids and malonyldialdehyde in erythrocytes from diabetic compared to erythrocytes from non-diabetic subjects. The degree of mem-

Table 2
Content of glucosylated PE in plasma and red blood cells of diabetics and control subjects as estimated on basis of total, diacyl and the palmitoyl-linoleoyl species of PE[a]

Subjects	% Total PE	% Diacyl PE	% 16:0–18:2 PE
Diabetics			
RBC	10.2 ± 2	18.4 ± 3	17.6 ± 2
Plasma	16.1 ± 3	27.7 ± 3	
Non-diabetics			
RBC	1.2 ± 0.5	5.6 ± 2	6.1 ± 4
Plasma	2.3 ± 1	1.5 ± 0.5	

[a]The percentages were calculated by expressing the sum of intensities in the various glucosylated species as a percentage of the total of each species or group or species. Means ± S.D. ($n = 4$).

brane lipid peroxidation was significantly correlated with the level of glycosylated hemoglobin.

Because of the apparent preferential glucosylation of the diacyl PE species, it would have been anticipated that the residual PE in the red blood cells of diabetics would be increased in plasmalogens. This was not obvious in the present experiments. Previously Freyburger et al. [17] had noted minimal amounts of dimethylacetals among the methylation products of the red blood cell PE. However, these workers also failed to detect the normal amounts of dimethylacetals in the PE of control subjects [17].

In view of random glycation of diacyl and alkenylacyl PE in vitro [7], the preferential glycation of the diacyl species of PE of the red blood cells and plasma observed in vivo in the present study requires an explanation. The phospholipid classes of the lipid bilayer of the red blood cell are known to be extensively segregated [19,20], and work with liposomes has demonstrated [21] that the sidedness of the free amino group influences the peroxidation of the aminophospholipids and their interaction with the secondary peroxidation products. Alternately, the diacyl and alkenylacyl species of PE might be subject to differential interaction with proteins, protecting the alkenylacyl and exposing the diacyl species of PE to glucosylation, regardless of their location in the bilayer. It is also possible that glucosylated alkenylacyl species of PE might have become preferentially deglucosylated by some of the defence mechanisms operating in the red blood cell membrane or plasma.

The failure to isolate glycated PE from the red blood cells of diabetics previously may have been due to the relatively easy dissociation of the Schiff base in the presence of dilute acid frequently employed in phospholipid recovery from TLC (e.g. organic solvents with 10% acetic acid). Using reference standards we had noted [7] that alkaline solvent systems were better suited than acid solvent systems for recovery of glycated PE from in vitro and in vivo samples. The mild electrospray ionization facilitated the detection of glycated aminophospholipids, which was not possible with fast atom bombardment mass spectrometry [7].

The glucosylation of a major proportion of the PE would be anticipated to affect the structure and function of the red cell membrane due to alterations in charge and the bulk of the glucosylated polar head group. It would also be expected to affect the susceptibility of the glucosylated phospholipid to phospholipases. We have reported elsewhere [13] that glucosylated PE is more susceptible to peroxidation than non-glucosylated PE, which is more resistant to peroxidation than PC [21]. Recently, Zommara et al. [22] have reported the inhibitory effect of ethanolamine plasmalogen on lipid peroxidation to be due to binding of iron and copper to liposomal lipids. It is therefore possible that hyperglycemia might play a role in promoting membrane lipid disorganization leading to peroxidation and atherosclerosis in diabetics.

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