Effect of Ethanol Amine Plasmalogens on Fe-Induced Peroxidation of Arachidonic Acid in Dipalmitoylphosphatidylcholine Vesicles

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We have investigated the influence of ethanolamine plasmalogens on iron-induced oxidation of arachidonic acid in dipalmitoylphosphatidylcholine (DPPC) vesicles. Lipoperoxidation was induced by the addition of 50 μM $FeSO_4$ and studied above (50 °C) and below (15 °C) the gel-to liquid transition temperature of the vesicles, at two different pH values (7.4 or 6.4). The extent of peroxidation was measured as thiobarbituric reactive product formed and the influence exerted by ethanolamine plasmalogens (PEPL) in this process was compared to that of dipalmitoylphosphatidylethanolamine (DPPE) and diacylphosphatidylethanolamines (DAPE). The extent of peroxidation of arachidonic acid embedded in DPPC vesicles was similar at the two temperatures and greater at 50 °C under acidic conditions. However, the peroxidative process was significantly decreased at 50 °C in the presence of PEPL, but not of DPPE or DAPE and the inhibitory effect was enhanced at pH 6.4. The possibility that a different phase distribution of the phospholipids, namely a transition from a lamellar to a hexagonal phase, may play a role in the scavenger effect of ethanolamine plasmalogens is discussed.

Key words plasmalogens; lipid peroxidation; phospholipids

Ethanolamine plasmalogens (1-alk-1-enyl-2-acylglycerophosphoetanolamines, PEPL) are glycerophospholipids that contain a vinyl ether bond in position *sn*-1 and an acyl bond in position *sn*-2. PEPL are particularly abundant in myelin, where 18 : 1 is the principal hydrocarbon chain in both the 1 and 2 positions.[1,2] This molecular species has been reported to be specific for nervous tissue[2] and changes have been observed in hibernation[3] and chronic alcohol consumption.[4] These findings led to the hypothesis that this particular lipid may represent a major factor in the homoviscous adaptation in the regulation of central nervous system membrane fluidity. Studies on Chinese hamster ovary mutant cells, deficient in peroxisomes and hence defective in plasmalogen biosynthesis, suggested that PEPL may play a role in the cellular protection against oxidation by reactive oxygen species.[5] This hypothesis was later supported by the observation that low density lipoproteins enriched with plasmalogens were strongly protected against oxidation.[6] On this basis, and taking into account that ethanol toxicity may be associated with generation of reactive O_2 species, it can be speculated that the increase of PEPL observed by different groups in alcoholic rats[4,7—8] might provide a molecular mechanism of defense against the ethanol-induced oxidative stress.

In the present study we investigated the influence of PEPL, purified from bovine brain phosphatidylethanolamine (PE), on iron-induced oxidation of arachidonic acid (AA) embedded in dipalmitoylphosphatidylcholine (DPPC) vesicles. The influence exerted by PEPL was compared with that of bovine brain diacylphosphatidylethanolamines (DAPE) and of the synthetic saturated form dipalmitoylphosphatidylethanolamine (DPPE).

MATERIALS AND METHODS

DPPC, bovine brain PE, DPPE, AA and 1,1,3,3-tetraethoxypropane and bathophenanthroline disulfonic acid (BPT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), silica gel plates (Kieselgel 60, HPTLC) from Merck Gmbh (Germany) and silicic acid (Biosil A, 200—400 mesh) from Biorad.

Isolation of PEPL and DAPE from Native Bovine Brain PE To isolate PEPL an aliquot (2—4 mg) of bovine brain PE was subjected to alkaline methanolysis in 3.5 ml of 0.1 M methanolic KOH according to Bergelson.[9] The solution was maintained at 0 °C for 30 min, cooled to −60 °C and neutralized by adding slowly and under stirring the proper amount of 5.7 M HCl. After addition of 2 vol of $CHCl_3$ and 20% water the sample was mixed and the two phases allowed to separate through a brief centrifugation. The lower phase was evaporated to dryness *in vacuo*, dissolved in $CHCl_3$ and subjected to chromatography at 4 °C on a column of silicic acid prepared in $CHCl_3$. Methylesters and unreacted PE were eluted by washing the column with 10 bed vol of $CHCl_3$ and 4 bed vol of $CHCl_3/CH_3OH$ (4 : 1, (v/v). PEPL were eluted in a further 8 bed vol of $CHCl_3/CH_3OH$ 4 : 1 (v/v), evaporated to dryness and resuspended in $CHCl_3/CH_3OH$ (2 : 1, v/v)

DAPE were isolated from total PE by exploiting the acid lability of the vinyl ether linkage. Briefly, total PE was dried under nitrogen and exposed to HCl fumes for 25 min. After 2 min of flushing with nitrogen, the sample was resuspended in methanol, neutralized with KOH 0.9 M in methanol and partitioned by the addition of 2 volumes of $CHCl_3$ and 20% water. DAPE recovered in the lower phase were purified from methylesters and unreacted PE by silicic column chromatography as described for PEPL. DAPE were eluted with 4 bed volumes of $CHCl_3/CH_3OH$ 4 : 1 (v/v), evaporated to dryness and resuspended in $CHCl_3/CH_3OH$ (2 : 1, v/v)

Calorimetric Measurements Measurements were performed using a Microcal (Amherst, MS) MC 2D calorimeter. The calorimetric data were automatically recorded and processed by an interfaced IBM PC computer and digitized by a DATA Translation DT 805 A/D converter. The measurements were performed as previously reported.[10]

Other Analyses The homogeneity of PEPL and DAPE

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was checked by HPTLC in the solvent system $CHCl_3$/CH_3OH/CH_3COCH_3/CH_3COOH/H_2O (50 : 15 : 15 : 10 : 5, by vol) performed before and after exposure of the sample to HCl fumes. Phospholipid concentration was determined according to Bartlett.[11]

The fatty acid composition of PEPL and DAPE was determined by capillary gas chromatography following derivatization in 0.5 M sodium methoxide in methanol.[12]

Preparation of Multilamellar Vesicles (MLVs) and Lipid Peroxidation Experiments MLVs composed of DPPC and 20% molar AA were used as the control and compared with vesicles containing, in addition, 5 mol% PEPL, DAPE or DPPE. Briefly, stock solutions containing known amounts of the different lipids were prepared in $CHCl_3$/CH_3OH 2 : 1 (v/v) and stored in the dark at −20 °C. Proper aliquots were mixed, taken to dryness under nitrogen, lyophilized overnight and resuspended in a proper volume of 25 mM Tris HCl buffer (pH 7.4), NaCl 154 mM in order to achieve a final DPPC concentration of 150 μM. Prior to the addition, the buffer was prewarmed at about 50 °C, *i.e.* at a temperature above the gel to liquid–crystalline transition temperature (Tm) assessed by DSC. $FeSO_4$ (50 μM final concentration) and other reagents were prepared and added to the buffer used for resuspension, immediately before the preparation of the vesicles. MLVs were incubated for 60 min above or below the gel to liquid transition temperature (50 °C or 15 °C respectively). The peroxidation reaction was stopped by adding 20 μl of 100 mM EDTA and measured as thiobarbituric reactive substances (TBARS) according to Buege and Aust,[13] using 1,1,3,3-tetraethoxypropane for the calibration curve.

Free Iron Determination The concentration of free Fe^{2+} was measured colorimetrically using bathophenantroline disulfonic acid (BPT) as chelator.[14] The amount of BPT-ferrous complex was determined by recording the optical density at 535 nm.

Table 1. Fatty Acid Composition of PEPL and DAPE Isolated from Bovine Brain PE

Fatty acid (%)	PEPL	DAPE
C16 : 0	2.0	6.9
C18 : 0	2.4	37.3
C18 : 1	61.8	27.7
C20 : 4	21.8	13.7
C22 : 6	11.9	14.3
S.I.[a]	0.02	0.26

a) S.I. was calculated by dividing the sum of percentages of saturated fatty acid by the sum of the unsaturated fatty acids times percent of the number of double bonds.

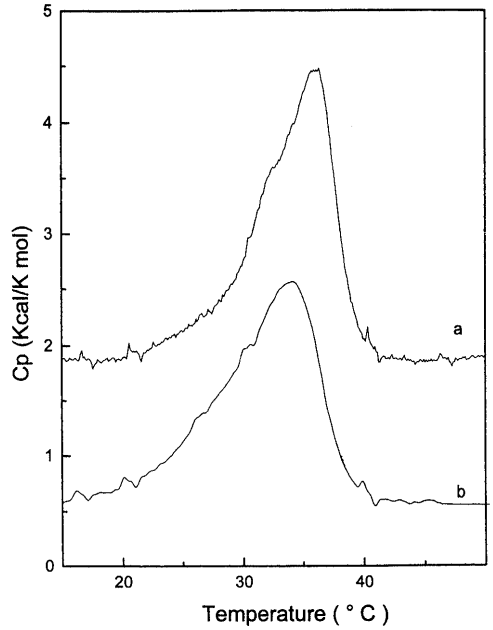

Fig. 1. Heat Capacity *vs.* Temperature for DPPC Vesicles
(a): DPPC/AA (20 mol%); (b): DPPC/AA (20 mol%)/PEPL (20 mol%).

RESULTS AND DISCUSSION

To characterize the PE molecular species used in the experiments and evaluate the influence of the vinyl ether moiety, purified PEPL and DAPE were analyzed for fatty acid composition (Table 1). In agreement with other reports,[15] a significantly higher proportion of C18 : 1 (61.8%) and a low proportion of C16 : 0 and C18 : 0 fatty acids have been found in PEPL compared to DAPE, leading to a lower saturation index (S.I.). Taking into account that the proportion of DAPE and PEPL in bovine brain PE is approximately 40 and 60%, respectively, the fatty acid composition of PEPL is in good agreement with that reported from Sigma for native PE (data not shown).

In Fig. 1 are reported the calorimetric scans for DPPC/AA and DPPC/AA/PEPL mixtures showing that PEPL added to DPPC/AA vesicles in a 1 : 1 molar ratio with AA, lower the main Tm from 36.0 to 34.0 °C. The same effect was observed in the presence of DAPE (data not shown). It has been shown that the presence of an ether linkage leads to an increase of the gel to liquid–crystalline phase transition temperature of plasmalogens compared to their diacyl form, because of a closer packing of lipids as well an increase in the strength of intermolecular hydrogen bonding between polar head groups.[16] Therefore, the observation that PEPL and DAPE exert the same effect on the thermotropic behavior of the DPPC/AA vesicles, indicates that the higher degree of unsaturation of PEPL partially counteracts the rigidifying effect of the ether bond.

Table 2 shows the extent of lipid peroxidation in DPPC/AA vesicles (control or containing 5 mol% PEPL, DPPE or DAPE) in two different conditions of temperature and pH. The extent of Fe^{2+}-induced peroxidation in control vesicles, evaluated at neutral pH, is similar at 50 and 15 °C, *i.e.* above and below the phase transition temperature; however, at acidic pH (6.4) the Fe^{2+}-induced TBARS production is significantly higher at 50 °C compared to 15 °C. These results can be explained by the structural organization of fatty acids in PC bilayers. In fact at neutral pH, fatty acids are present in PC bilayers as patches, *i.e.* they are segregated into clusters.[17] Fatty acid clustering occurs both above and below the phase transition of DPPC but intermolecular hydrogen bondings, increasing at lower pH, might be important in stabilizing the fatty acid aggregates[17] where lipid peroxidation is promoted. It cannot be excluded, however, that the greater solubility of iron under acidic conditions might also contribute to the higher extent of peroxidation at pH 6.4.

Further experiments showed that the peroxidation of AA in DPPC vesicles is unaffected by the addition of mannitol, a

Table 2. Effect of pH and Temperature on the Peroxidation of DPPC/AA Vesicles Containing 5 mol % DPPE, DAPE or PEPL

	pH 7.4		pH 6.4	
	15 °C	50 °C	15 °C	50 °C
DPPC/AA	280± 7	308±4	275±10	386± 9♦
DPPC/AA/DPPE	275± 7	300±9	297± 7	430± 6*
DPPC/AA/PEPL	314± 6	208±6*	403± 8*	111± 3*
DPPC/AA/DAPE	320±10	320±8	410± 7*○	460±10*

Samples were incubated for 60 min in the presence of 50 μM $FeSO_4$. Data are expressed as nmol TBARS/μmol AA (mean±S.D. of triplicate determinations). * $p<0.01$ *vs.* DPPC/AA vesicles under the same conditions of pH and temperature. ♦ $p<0.05$ *vs.* DPPC/AA vesicles at pH 7.4, 50 °C. ○ $p<0.01$ *vs.* DPPC/AA/DPPE vesicles at pH 6.4, 15 °C.

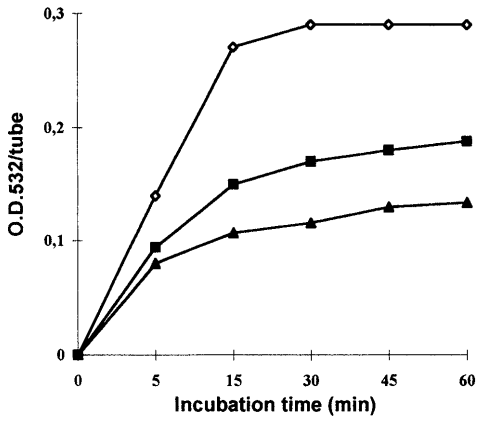

Fig. 2. Kinetic of Peroxidation of AA

(-◇-◇-): in DPPC vesicles; (■-■-): in DPPC vesicles containing 10 μM (5% molar) PEPL; (▲-▲-): in DPPC vesicles containing 20 μM (10% molar) PEPL. Samples were incubated at 50 °C in the presence of 50 μM $FeSO_4$.

powerful OH• scavenger (data not shown). This finding supports the hypothesis that AA peroxidation is not dependent on the production of OH• radicals.[18,19] On the other hand, the capability of DPPC/AA vesicles for binding iron (about 1 nmol/4 nmol AA, data not shown) raises the possibility that the main driving force of AA peroxidation might be the iron-catalyzed decomposition of contaminant hydroperoxides (LOOH) to alkoxyl radicals (LO•).[19] Rao *et al.*[20] and Peterson *et al.*[21] suggested that, in the presence of oxygen, iron may form an unstable complex with polyunsaturated fatty acids that involves a hydroperoxide and promotes Fe^{2+} oxidation, leading to a Fe^{2+}/Fe^{3+} molar ratio more favorable to lipid peroxidation.[22]

The three molecular species of PE exert different effects on the peroxidation of DPPC/AA vesicles, evaluated at 15 and 50 °C (Table 2). DPPE and DAPE behave similarly at 50 °C. However, different effects on AA peroxidation are remarkable at 15 °C. At neutral pH, a small increase in AA peroxidation can be observed in vesicles containing DAPE. This effect is strongly enhanced at acidic pH, which is under conditions where intermolecular hydrogen bondings are important in promoting peroxidation. These results suggest a contribution of the fatty acid composition of DAPE and DPPE. In fact, the fluidization promoted at 15 °C by the high degree of unsaturation of DAPE can play a critical role in promoting the peroxidative processes when the bilayer is in the gel state. On the contrary, at 50 °C when the bilayer is in the fluid state, the contribution of DAPE and DPPE fatty acid composition is comparable.

On the contrary, PEPL significantly decrease AA peroxidation at 50 °C. The inhibitory effect at 50 °C, pH 7.4, is further confirmed by the kinetic data reported in Fig. 2, showing the time course of Fe^{2+}-induced AA peroxidation in DPPC/AA vesicles containing two different PEPL concentrations (10 or 20 μM, *i.e.* 5 or 10 mol %, respectively). At 15 °C, PEPL do not reduce the extent of AA peroxidation. Considering the possibility that PEPL might influence the AA peroxidation by affecting the binding of iron to the vesicles, we evaluated the amount of free iron in vesicles of DPPC/AA or DPPC/AA containing different PEPL proportions, showing no differences.

The antiperoxidative effect exerted by PEPL only at the higher temperature, suggests the possibility that PEPL and AA may interact or adopt a peculiar molecular packing only in the liquid–crystalline state of DPPC bilayer. In this regard, it is possible that the greater capability of PEPL, compared to DPPE and DAPE, to form hexagonal phases plays a role in the rate of AA peroxidation. The temperature at which the lamellar/hexagonal phase transition occurs, appears in fact dependent on the degree of PE unsaturation[16] and C18 : 1 fatty acid content.[23] Moreover, it has been reported that the plasmalogen form of phosphatidylethanolamine undergoes the L/H transition at a significantly lower temperature than the diacyl analog.[16,24—26] The ether linkage, causing a small increase in the strength of intermolecular interactions at the head group region, together with the double bond between the first and second carbon of the alk-1-enyl chain, increasing the molecular volume of the hydrocarbon region, destabilize the lamellar phase and promote the transition to the HII phase. Worth noting is that the ability of PEPL to form non bilayer structures, well established in vesicles constituted solely or primarily of PEPL, has also been shown in DPPC vesicles containing low PEPL concentrations (2—8 mol %).[27] No data are available in the literature concerning the possibility that, in DPPC vesicles containing AA, changes induced by AA in the physical properties of bilayers may prevent HII phase. This feature therefore deserves further investigation. However, the higher bilayer fluidity induced by AA should be a factor favoring, rather than preventing, HII phase.[25,28]

The possibility that the antiperoxidative activity of PEPL may be dependent on a different lamellar/hexagonal distribution is supported by the two following observations: 1) at 50 °C, the antiperoxidative effect is potentiated at pH 6.4. At acidic pH the area/lipid molecule in the polar region decreases, therefore favoring the intermolecular hydrogen bonding between the polar head-groups of PEPL and consequently the formation of hexagonal phases;[26] 2) at either pH value, no antiperoxidative effect is observed at 15 °C, *i.e.* when the bilayer is in the gel state. X-ray studies have pointed out that in the gel state no destabilization in HII phase occurs and phospholipids are always present in the lamellar configuration, presumably due to packing constraints in the hydrocarbon region.[25,28] On the contrary, as the temperature is raised, the increased volume of the hydrocarbon region and the lack of expansion at the head-group region induce the shift to the hexagonal phase. Worth noting, at 15 °C in the acidic conditions, PEPL-(as well DAPE-) containing vesicles show a greater TBARS production compared to control or DPPE-containing vesicles, possibly due to an

enhancement of AA phase separation when these lipids are in the gel state.

However, we cannot deny that the peculiar fatty acid composition of PEPL may play a critical role in the scavenger effect. Certainly, in a more fluid microenvironment due to the higher degree of unsaturation, than in DAPE-vesicles, active movements of PEPL within membranes and contact between PEPL and the radical centered lipids could be favored, therefore facilitating the PEPL scavenger effect.

In conclusion, our experimental evidence shows that peroxidation events involving polyunsaturated fatty acids can be modulated that is, either enhanced or inhibited, by varying factors such the temperature or pH which influence the phase distribution of vesicle phospholipids. In DPPC/AA model membranes the shift from lamellar to an inverse hexagonal lipid structure, induced by PEPL, seems to contribute to the scavenger effect of PEPL. It is not excluded that also in natural membranes, as well in lipoproteins, this mechanism may partially contribute to the protective role of PEPL.

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