Delayed oxidative degradation of polyunsaturated diacyl phospholipids in the presence of plasmalogen phospholipids *in vitro*

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The oxidative degradation of plasmalogen (alkenylacyl) phospholipids was analysed in the absence and the presence of polyunsaturated ester phospholipids by ^1H -NMR and by chemical determination. Brain lysophosphatidylethanolamine (lyso-P-PE), brain P-PE and erythrocyte P-PE, containing an increasing number of intrachain double bonds at sn_2 , were oxidized with 2,2'-azobis-(2-amidinopropane hydrochloride) (AAPH; 2 or 10 mM) in Triton X-100 micelles (detergent/phospholipid 1:5, mol/mol). The formation of two peroxy radicals was accompanied by the degradation of approx. one molecule of brain lyso-P-PE. On oxidation of brain P-PE or erythrocyte P-PE (320 nmol) with 2 mM AAPH, the (α -vinyl) methine ^1H signal of the enol ether decreased more rapidly than the methine proton peak of intrachain double bonds. The rate of enol ether degradation increased in the order: erythrocyte P-PE > brain P-PE > brain lyso-P-PE. The disappearance of the polyunsaturated ester phospholipids 1-palmitoyl-2-arachidonoyl phosphatidylcholine (16:0/20:4-PC) and 1-palmitoyl-2-linoleoyl phosphatidylcholine

(16:0/18:2-PC) (100 nmol), as induced by 10 mM AAPH, was nearly completely inhibited by the plasmalogens (25 nmol) in the first 30 and 60 min of incubation respectively, and was delayed at later time points. Plasmalogens and vitamin E (4–25 nmol) mitigated the decreases in 16:0/[^3H]20:4-PC (100 nmol) induced by 2 mM AAPH in a similar manner. The initial rate of degradation of intrachain double bonds of 16:0/20:4-PC and 16:0/18:2-PC (320 nmol; 2 mM AAPH) was decreased by 59 % and 81 % respectively in the presence of 80 nmol of brain lyso-P-PE. In conclusion, plasmalogens markedly delay the oxidative degradation of intrachain double bonds under *in vitro* conditions. Interactions of enol ether double bonds with initiating peroxy radicals as well as with products generated by prior oxidation of polyunsaturated fatty acids are proposed to be responsible for this capacity of plasmalogens. Furthermore, the products of enol ether oxidation apparently do not propagate the oxidation of polyunsaturated fatty acids.

INTRODUCTION

The peroxidation of lipids is generally thought to play a prominent role in the pathogenesis of atherosclerosis, as the oxidative modification of lipid components of lipoproteins most probably promotes atherogenesis in its early phase (for reviews, see [1,2]). At later stages of the atherosclerotic process, e.g. during coronary ischaemia and reperfusion injury, lipid peroxidation appears to contribute to the perturbation of cellular membrane structure [3]. Consequently, there is great interest in lipid-soluble molecules that could reinforce the oxidative resistance of lipoproteins and cellular membranes against peroxidation damage.

Several reports indicate a particular sensitivity of plasmalogen (alkenylacyl) phospholipids to attack by oxygen radicals, which is believed to be related to the presence of the enol ether double bond of alkenyl chains at the C-1 atom. Plasmalogen deficiency of pyrene-labelled CHO cells was shown to be associated with an increased cytotoxicity upon irradiation with UV light, whereas restoring normal plasmalogen levels increased the resistance of the cells to the cytotoxic effect of UV treatment [4]. Enrichment of low-density lipoproteins (LDLs) with different types of plasmalogens delays the formation of conjugated double bonds induced by various oxidants [5,6], indicating that plasmalogens are able to suppress the peroxidation of LDL-associated fatty acids.

In LDL particles, the content of plasmalogen phospholipids is approx. 30 $\mu\text{mol}/\text{mmol}$ of LDL cholesterol [7]. The percentage of plasmalogens in membranes of animal cells ranges in general between 15 and 20 % of total phospholipids, but can reach more than 50 % in the sarcolemma of some species [8]. The plasmalogen phospholipids found in cellular membranes and lipoproteins predominantly contain ethanolamine and choline as headgroups [plasmalogen (P-PE) and plasmalogen (P-PC)].

Lipid peroxidation is generally assumed to start from oxidative modification of the double bonds of polyunsaturated fatty acids. In the first step of oxidation of these intrachain double bonds, peroxy radicals are thought to abstract a hydrogen atom from the bisallylic methylene group located between two adjacent double bonds of the hydrocarbon chain [9,10]. This results in the formation of conjugated double bonds. Reaction of the fatty acid radical with oxygen may yield fatty acid peroxy radicals, which are able to abstract a hydrogen atom from the methylene group of another polyunsaturated fatty acid. Thus the oxidizing fatty acid is transformed into a hydroperoxide. This, in turn, may be degraded further into products such as aldehydes. As the oxidation of the polyunsaturated fatty acids confers instability on the hydrocarbon chains (e.g. by the formation of conjugated double bonds), their decomposition into fragments is facilitated [9,10].

In order to gain more insight into the conditions under which plasmalogen phospholipids may delay lipid peroxidation, in the

Abbreviations used: LDL, low-density lipoprotein; P-PE, plasmalogen phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; AAPH, 2,2'-azobis-(2-amidinopropane hydrochloride); TX-100, Triton X-100; 16:0/18:2-PC, 1-palmitoyl-2-linoleoyl phosphatidylcholine; 16:0/20:4-PC, 1-palmitoyl-2-arachidonoyl phosphatidylcholine.

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present study the oxidation of plasmalogen phospholipids was induced by the water-soluble azo initiator 2,2'-azobis-(2-amidinopropane hydrochloride) (AAPH) in the presence or the absence of different types of polyunsaturated ester phospholipids. The early phase of oxidative degradation of polyunsaturated fatty acids was analysed using $^1\text{H-NMR}$ by measuring the amplitudes of methine proton signals attributable to intrachain and enol ether double bonds. This method has previously been applied to assess qualitative changes induced by the oxidation of fatty acids [11,12]. In parallel, oxidative degradation was also monitored by assessing the disappearance of the oxidizable substrate using TLC. In this way, extensive degradation of the polyunsaturated phospholipids is monitored. With few exceptions, in previous studies the capacity of plasmalogens to delay lipid peroxidation was investigated in tissue preparations, whole cells or lipoproteins. The complexity of those systems renders the interpretation of the data difficult as, for example, a great variety of different molecules may be oxidized in parallel. In the present study, experiments were performed using detergent micelles containing ester and plasmalogen phospholipids of known hydrocarbon composition. This system allows us to register the oxidative degradation of phospholipids under on-line conditions by $^1\text{H-NMR}$.

Interestingly, in most animal cell types and lipoproteins so far analysed, major portions of P-PE and plasmenylcholine are esterified at the C-2 atom with highly polyunsaturated fatty acids. For example, in human plasma lipoproteins and in total membranes of erythrocytes and platelets, 75–90% of molecular species of P-PE molecules contain fatty acids with four or more double bonds at sn_2 [13–15]. Thus the question arises as to whether the presence of these highly polyunsaturated fatty acids within the plasmalogen molecules contributes to the repeatedly demonstrated high sensitivity of plasmalogen phospholipids to oxidation. In order to address this point, the degradation of ester phospholipids containing polyunsaturated fatty acids and/or of plasmalogen phospholipids was analysed using alkenylacyl phospholipids containing on average either four (erythrocyte P-PE) or two (bovine brain P-PE) double bonds within the hydrocarbon chain linked to C-2. Furthermore, the oxidative degradation of a lysoplasmalogen containing no double bond at sn_2 (brain 2-lyso-P-PE) was also analysed.

MATERIALS AND METHODS

Materials

1-Hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine [1-palmitoyl-2-linoleoyl phosphatidylcholine (16:0/18:2-PC)], 1-hexadecanoyl-2-[(*cis,cis,cis,cis*)-5,8,11,14-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine [1-palmitoyl-2-arachidonoyl PC (16:0/20:4-PC)], 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine from soy bean [soy bean phosphatidylethanolamine (PE)], 1-monoacyl-*sn*-glycero-3-phosphocholine from egg yolk (egg 2-lyso-PE), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine from bovine brain (brain P-PE), 1,2-diacyl-*sn*-glycero-3-phosphocholine from bovine heart (heart PC), 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk (egg PC), 1-monoacyl-*sn*-glycero-3-phosphocholine from egg yolk (egg 2-lyso-PC) and 1-hexadecanoyl-*sn*-glycero-3-phosphocholine (1-palmitoyl-2-lyso-PC) were obtained from Sigma (Deisenhofen, Germany). The purity of all commercially obtained phospholipids was assessed by one-dimensional TLC according to procedure 1 (see below). 1-Palmitoyl-2-[5,6,8,9,11,12,14,15- ^3H]-arachidonoyl PC was synthesized from 1-palmitoyl-2-lyso-PC and [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (Amersham, Braunschweig, Germany) as described in [16]. 1-Alkenyl-2-lyso-PC, 1-

alkenyl-2-lyso-PE and 1-alkenyl-2-acyl-PE were prepared from bovine heart PC and from bovine brain PE by alkaline methanolysis [17]. The purity of the products, as determined by exposure to HCl fumes for 3–6 min, was between 93 and 95%. A detailed description of the TLC procedure used to determine the plasmalogen content of phospholipids has been given elsewhere [7].

1-Alkenyl-2-acyl-PE was also isolated from human erythrocytes. Blood from apparently healthy donors was centrifuged and plasma as well as buffy coats discarded. The lipids from erythrocytes were extracted [18], phospholipids were separated by one-dimensional TLC using the solvent chloroform/methanol/ NH_3 /water (90:54:5.5:5.5, by vol.), the spot corresponding to PE was extracted with chloroform/methanol (1:4, v/v), and the ethanolamine phospholipids were subjected to alkaline methanolysis. The purity of erythrocyte P-PE thus isolated was 93%. All isolation procedures were performed under nitrogen. Tubes were only opened while pipetting. For storage of the phospholipids, the solutions were kept at -20°C under argon in closed tubes.

Triton X-100 (TX-100) was from Serva (Heidelberg, Germany). AAPH was provided by Polysciences Inc. (Eppelheim, Germany).

Assessment of phospholipid degradation by TLC and phosphate determination

Usually, 100–125 nmol of unlabelled or ^3H -labelled phospholipids (plasmalogen and/or ester phospholipids) was dissolved in chloroform/methanol (2:1, v/v) and evaporated. A 500 nmol portion of TX-100 (or, in some experiments, 1-palmitoyl-2-lyso-PC) was dispersed in 0.5 ml of a Hepes buffer containing 135 mM NaCl and 15 mM Hepes (pH 7.4) or in double-distilled water. The TX-100 suspension was added to the dried phospholipids. After vortexing for 10 s, the mixture was incubated at 37°C for up to 6 h in the presence or absence of different concentrations of AAPH. After the end of the incubation, the suspensions were separated into two phases by the method of Bligh and Dyer [19] by using chloroform that contained 50 mg/l butylated hydroxytoluene. The lower organic phase and, in some experiments, also the upper phase were analysed for phospholipids and their degradation products on Merck DC 60 plates.

The following TLC procedures (indicated in legends to the Figures) were employed: procedure 1, one-dimensional, solvent chloroform/methanol/ NH_3 /water (90:54:5.5:5.5, by vol.); procedure 2, two-dimensional, solvent chloroform/methanol/acetic acid (65:25:10, by vol.) in the first direction and chloroform/methanol/formic acid/water (65:25:8.9:1.1, by vol.) in the second direction; procedure 3, one-dimensional, solvent water/methanol/ NH_3 (70:48:7, by vol.). Usually, lipids were visualized by exposure to iodine vapour, and the spots were scraped off the plate and quantified by determining their phosphate content [20]. In the case of ^3H -labelled phospholipids, radioactivity was determined as described in [19] in the upper as well as the lower phase by using a scintillation counter.

Assessment by $^1\text{H-NMR}$ spectroscopy of oxidative modification of acyl and alkenyl chains

Samples of 320–400 nmol of phospholipids were dissolved in chloroform/methanol (2:1, v/v). The bulk of the solvent was removed by a stream of argon and the remainder was dried under vacuum overnight. A 5-fold molar excess of TX-100 (or of 1-palmitoyl-2-lyso-PC) dissolved in 0.75 ml of $^2\text{H}_2\text{O}$ was added and the mixture was vortexed under argon for 30 s. The clear solution was transferred into a 5 mm outer-diam. NMR sample

Table 1 Average number of intrachain double bonds of hydrocarbon chains linked to the C-1 and C-2 atoms of plasmalogen (alkenylacyl-) phospholipids and diacyl phospholipids

The molecular species composition of the phospholipids was analysed as described in the Materials and methods section.

Phospholipid	Average number of intrachain double bonds	
	C-1 chain	C-2 chain
Plasmalogen phospholipids		
Brain P-PE	0.21	2.23
Erythrocyte P-PE	0.18	3.71
Brain plasmalogencholine	0.09	2.55
Diacyl phospholipids		
Soy bean PE	1.05	2.17
Egg PE	0.14	2.29
16:0/20:4-PC	0	4.0
16:0/18:2-PC	0	2.0
Egg PC	0.02	1.93

tube and lipid oxidation was initiated by adding the oxidant directly into the tube. $^1\text{H-NMR}$ spectra were recorded at 37 °C using a Varian 400S spectrometer operating at 400 MHz. Typically, 96 transients were recorded with a total relaxation delay of 5 s. Suppression of the residual water signal was achieved by a 1.5 s low-power decoupler pulse. Signal assignments were made in a double-quantum filtered two-dimensional shift correlated spectrum (COSY). Chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulphonic acid. Oxidative degradation of intrachain double bonds, enol ether double bonds and bisallylic methylene groups (located between two adjacent intrachain double bonds) was followed by measuring the peak amplitudes of the respective methine and methylene proton signals over a time range of 0–180 min.

Other analyses

Molecular species analysis of phospholipids was performed by phospholipase C treatment of phospholipids, derivatization of diradylglycerols with 2,3-dinitrobenzoylchloride, separation of individual species by HPLC and detection of the absorbance of the peaks at 254 nm as described in detail previously [21,22]. The vitamin E content of micelles was measured using a HPLC procedure as detailed in [5].

RESULTS

Three different ethanolamine plasmalogen phospholipids were prepared: 1-alkenyl-2-lyso-PE (brain lyso-P-PE) and two types of 1-alkenyl-2-acyl PEs, obtained from bovine brain and from human erythrocytes. Brain P-PE and erythrocyte P-PE contained on average 2.2 and 3.7 intrachain double bonds in the acyl chains esterified to C-2 (Table 1). The alkenyl chains present at C-1 contained on average 0.2 intrachain double bonds. Thus the three types of ethanolamine plasmalogens differed mainly in the number of intrachain double bonds of the acyl chains at C-2.

Samples of 100 nmol of each of the three plasmalogens were incorporated into TX-100 micelles and incubated with a concentration of 2 or 10 mM of the water-soluble oxidant AAPH which, in the presence of oxygen, produces peroxy radicals at a constant rate [23]. Incubation with the oxidant led to a decrease in plasmalogen phospholipids (Figure 1). Without AAPH, the decrease was between 6.1 % and 10.4 % after 360 min. The decrease in brain lyso-P-PE induced by AAPH was accompanied by an increase in the formation of a substance in the water phase that co-migrated with glycerol-PE (Figure 1, left panel). Glycerol-PE is most probably generated by the decomposition of the vinyl ether double bond present in the C-1 chain, together with long-chain aldehydes [23,24]. The amount of glycerol-PE generated was almost exactly equal to the quantity of brain lyso-P-PE degraded by the two concentrations of AAPH. After 360 min of incubation, 19.7 % (2 mM AAPH) and 73.3 % (10 mM AAPH) of brain lyso-P-PE was lost.

Assuming a constant rate of free-radical generation from AAPH decomposition of $1.36 \times 10^{-6} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}^{-1}$ [25], it can

Figure 1 Time-dependence of the effect of AAPH on plasmalogen phospholipids and formation of degradation products

Portions of 100 nmol of brain lyso-P-PE (2-lyso BP-PE), brain P-PE (BP-PE) or erythrocyte P-PE (EP-PE), enriched in TX-100 micelles (500 nmol) dispersed in 0.5 ml of Hepes buffer, were treated for 0–360 min with 2 or 10 mM AAPH at 37 °C. After different time intervals, the suspensions were separated into two phases [19]. Lipids in the lower (organic) phase were separated by one-dimensional TLC using procedure 1 (see the Materials and methods section). The upper phase was separated by one-dimensional TLC using procedure 3. Empty symbols, 2 mM AAPH; filled symbols, 10 mM AAPH. GPE, glycerol-PE. Results are means $\pm$ S.E.M. of between four and eight experiments, or means of two independent experiments (no bars shown).

Figure 2 Kinetics of the effect of AAPH on peak amplitudes of (α -vinyl) methine ^1H and olefinic intrachain double bond methine ^1H of plasmalogen phospholipids as detected by ^1H -NMR

The chemical shifts of the enol ether methine ^1H and of intrachain methine protons were identified at 6.0 p.p.m. and 5.4 p.p.m. respectively. Portions of 320 nmol of brain lyso-P-PE (lyso BP-PE), brain P-PE (BP-PE) or erythrocyte P-PE (EP-PE) were enriched in TX-100 micelles (1600 nmol) dispersed in 0.75 ml of $^2\text{H}_2\text{O}$ and treated for up to 180 min with 2 mM AAPH at 37 °C. The values obtained at 0 min were set as 100%. Upper panel: degradation of enol ether methine protons of the alkenyl chains at δ_{H} . Lower panel: oxidative degradation of intrachain methine protons. Data are from one representative out of three or four independent experiments.

be calculated using the values of brain lyso-P-PE degradation and of glycerol-PE formation after 120 min (as determined from the experiments shown in Figure 1) that, for each 1 nmol of peroxyl radical formed, 0.48–0.56 nmol of brain lyso-P-PE disappeared upon oxidation with 2 and 10 mM AAPH. Similar calculations for the amounts of brain lyso-P-PE and glycerol-PE assessed after 180 min of incubation yielded values of 0.43–0.51 nmol.

The kinetics of the decreases in alkenylacyl phospholipids elicited by AAPH (Figure 1, middle and right panels) were relatively similar to those observed with brain lyso-P-PE as substrate. After 360 min of incubation, 20.6% and 20.5% (2 mM AAPH) and 77.3% and 73.1% (10 mM) of brain P-PE and erythrocyte P-PE respectively had disappeared. When micelles containing brain P-PE were dispersed in double-distilled water (instead of Hepes buffer), 25.5% and 75.9% was lost after 360 min with 2 and 10 mM AAPH respectively. The decrease in brain P-PE was accompanied by the appearance of a spot comigrating with lyso-PE. In order to differentiate between the 1-lyso and 2-lyso isoforms of the degradation product, two-dimensional TLC (procedure 2; see the Materials and methods section) was employed. Of the total lyso-PE obtained by oxi-

Figure 3 Time-dependence of the effect of plasmalogen phospholipids on the oxidative degradation of 16:0/20:4-PC

Portions of 100 nmol of 16:0/20:4-PC (enriched in 1600 nmol of TX-100 micelles dispersed in 0.5 ml of Hepes buffer) without or with 25 nmol of brain lyso-P-PE (lyso BP-PE), brain P-PE (BP-PE), erythrocyte P-PE (EP-PE) or soy bean PE (SB-PE) were treated for up to 360 min with 10 mM AAPH at 37 °C. After various time intervals, lipids were extracted [19] and phospholipids were separated by two-dimensional TLC (procedure 2). Upper panel: degradation of 16:0/20:4-PC; lower panel: concomitant decomposition of added plasmalogen phospholipids. Results are means $\pm$ S.E.M. of four or means of two (where no bars are shown) independent experiments.

dation of brain P-PE with 2 mM AAPH, 94% consisted of 1-lyso-PE, with the remaining portion being formed by 2-lyso-PE. Lyso-PE was also the major degradation product identified upon oxidation of brain P-PE and erythrocyte P-PE with 10 mM AAPH.

Samples of 320 nmol of brain lyso-P-PE, brain P-PE and erythrocyte P-PE present in TX-100 micelles were oxidized for up to 180 min with 2 mM AAPH. The peak amplitudes of the enol ether protons of the three plasmalogens decreased time-dependently in the presence of the oxidant, as assessed by ^1H -NMR (Figure 2, upper panel). The amplitudes of the TX-100 signals at 6.8 p.p.m. and 7.2 p.p.m. routinely measured as a control were unchanged within the incubation period of 180 min (results not shown).

Within the first 90 min, the peak amplitudes of enol ether methine ^1H signals decreased at rates of 8.4%/h (brain lyso-P-PE), 9.2%/h (brain P-PE) and 10.0%/h (erythrocyte P-PE). When 0.1 mM EDTA was added to the micellar suspensions, the degradation rate of the enol ether methine ^1H of brain P-PE was 9.9%/h. At longer incubation times, the more rapid decrease in the enol ether ^1H peak from erythrocyte P-PE as compared with those from brain P-PE and brain lyso-P-PE was somewhat more evident (Figure 2, upper panel). The amplitudes of methine proton signals of intrachain double bonds in brain P-PE and erythrocyte P-PE were not altered in the first 30 min of incubation

Figure 4 Concentration-dependence of the effect of plasmalogen phospholipids on the oxidative degradation of 16:0/20:4-PC

Portions of 100 nmol of 16:0/20:4-PC (enriched in 500 nmol of TX-100 micelles dispersed in Hepes buffer) without or with different amounts of the indicated plasmalogens, ester phospholipids or vitamin E were treated for 360 min with 2 mM AAPH at 37 °C. Thereafter, lipids were extracted [19] and phospholipids were separated by two-dimensional TLC (procedure 2). When 25 nmol of added substances was present at time zero, the amounts of the substances had decreased to 8.2 nmol [brain P-PE (BP-PE)], 16.9 nmol [soy bean PE (SB-PE)] and 13.0 nmol (vitamin E) after 360 min. With 8 nmol of added substances initially, the quantities had decreased to 2.1 nmol (brain P-PE), 5.6 nmol (soy bean PE) and 1.4 nmol (vitamin E) after 6 h. Results are means $\pm$ S.E.M. of four independent experiments.

(Figure 2, lower panel). Thereafter, these amplitudes started to decrease slowly, with a decrease of 13–16 % being observed after 180 min. Comparable decreases were seen after 180 min for the peak amplitudes of the bisallylic methylene ^1H signals of brain P-PE and erythrocyte P-PE (results not shown).

Micelles were enriched in diacyl phospholipids and plasmalogens in a ratio of 4:1 (mol/mol), a phospholipid ratio encountered in several biological membranes (see the Introduction). Chemical determinations using TLC indicated that the amount of the polyunsaturated PC species 16:0/20:4-PC was rapidly lowered in the presence of 10 mM AAPH in the first 120 min of

incubation, with a rate of 13.9 nmol/h. A somewhat slower decrease was observed at incubation times > 120 min (Figure 3, upper panel). When micelles were composed of 1-palmitoyl-2-lyso-PC instead of TX-100, the amount of PC was decreased to 24.8 nmol by 10 mM AAPH after 360 min.

Addition of 25 nmol of plasmalogens to TX-100 micelles containing 100 nmol of 16:0/20:4-PC completely inhibited degradation of the ester phospholipid within the first 30 min of incubation. Thereafter, the amount of the PC species started to decline. After 180 min, the degradation of 16:0/20:4-PC was decreased by 46–48 % by the presence of plasmalogen phospholipids (Figure 3, upper panel). At the same time point, plasmalogen phospholipids were decreased to 10 % or less of initial values (Figure 3, lower panel). The degradation rate of the plasmalogens was 6.2 nmol/h (brain lyso-P-PE) and 7.1 nmol/h (brain P-PE, erythrocyte P-PE) between 0 and 180 min. Addition of 25 nmol of the polyunsaturated soy bean PE did not affect the loss of the 16:0/20:4-PC (Figure 3, upper panel).

In the presence of 2 mM AAPH, the amount of 16:0/20:4-PC was decreased from 100 to 78.9 nmol after 360 min (Figure 4). Addition of increasing proportions of brain P-PE (or of brain lyso-P-PE; results not shown) diminished the degradation of PC. In contrast, addition of soy bean PE barely affected the oxidative degradation of PC. In the presence of vitamin E, the oxidative loss of the PC species was clearly diminished (by 73 % at 25 nmol of vitamin E; Figure 4). The concomitant decreases in the amounts of the added substances are indicated in the legend to Figure 4 (25 nmol present at time zero).

The results of experiments using 16:0/[^3H]20:4-PC-containing micelles as the oxidizable substrate are shown in Table 2. [^3H]PC radioactivity (present in the organic phase after extraction by the method of [19]) was decreased from 12060 d.p.m. (time zero) to 10760 ± 980 d.p.m. (2 mM AAPH) and 9080 ± 490 d.p.m. (10 mM AAPH) after 120 min, and to 10160 ± 880 d.p.m. (2 mM AAPH) and 7720 ± 1040 d.p.m. (10 mM AAPH) after 180 min (means $\pm$ S.E.M., $n = 3$). Following a 360 min incubation, PC-associated ^3H radioactivity was lowered by 24 % (2 mM AAPH) and 72 % (10 mM AAPH) (Table 2). All radioactivity present in the organic phase was associated with the PC spot (results not shown). After 360 min, 11 % (2 mM AAPH) and 12 % (10 mM AAPH) of total radioactivity was recovered in the upper aqueous

Table 2 AAPH-induced degradation of 16:0/[^3H]20:4-PC

The substrate 16:0/[^3H]20:4-PC was enriched in TX-100 micelles (500 nmol) dispersed in double-distilled water. The micelles contained, in addition, 100 nmol of egg PC. After incubation with AAPH, the micellar suspension was separated into organic and aqueous phases [19]. The radioactivity was determined in the TLC spot corresponding to PC (after separation of the organic phase by procedure 1; see the Materials and methods section) and in the aqueous phase. Results are means $\pm$ S.E.M. of three or four means of two independent experiments. n.d., not determined.

	PC-associated ^3H (d.p.m.)		^3H in aqueous phase (d.p.m.)	
	2 mM AAPH	10 mM AAPH	2 mM AAPH	10 mM AAPH
0 min	12060 $\pm$ 1180	12060 $\pm$ 1180	0	0
360 min	9210 $\pm$ 480	3380 $\pm$ 270	320 $\pm$ 60	1040
360 min				
+ Brain lyso-P-PE (4 nmol)	9680 $\pm$ 840	4430 $\pm$ 580	240 $\pm$ 30	700
+ Brain lyso-P-PE (8 nmol)	10060 $\pm$ 760	6580 $\pm$ 220	210 $\pm$ 20	640
+ Brain lyso-P-PE (25 nmol)	10770 $\pm$ 520	8710 $\pm$ 590	180 $\pm$ 30	290
+ Brain plasmalogen (25 nmol)	11040 $\pm$ 840	9080 $\pm$ 710	160 $\pm$ 10	250
+ Brain P-PE (8 nmol)	10110 $\pm$ 1230	7310 $\pm$ 820	240 $\pm$ 40	n.d.
+ Brain P-PE (25 nmol)	11240 $\pm$ 1170	9410 $\pm$ 880	190 $\pm$ 20	n.d.
+ Egg lyso-PE (25 nmol)	9650 $\pm$ 790	4600 $\pm$ 550	340 $\pm$ 50	1020
+ Egg lyso-PC (25 nmol)	9080 $\pm$ 1020	4520 $\pm$ 350	290 $\pm$ 40	890
+ Soy bean PE (25 nmol)	10030 $\pm$ 770	4910 $\pm$ 630	300 $\pm$ 20	1290

Figure 5 Time-dependence of the effect of plasmalogen phospholipids on the oxidative degradation of 16:0/18:2-PC

Portions of 100 nmol of 16:0/18:2-PC (enriched in 500 nmol of TX-100 micelles dispersed in Hepes buffer) without or with 25 nmol of brain lyso-P-PE (lyso BP-PE), brain P-PE (BP-PE), erythrocyte P-PE (EP-PE) or soy bean PE (SB-PE) were treated for up to 360 min with 10 mM AAPH at 37 °C. After various time intervals, lipids were extracted [19] and phospholipids were separated by two-dimensional TLC (procedure 2). Upper panel: degradation of 16:0/18:2-PC; lower panel: concomitant decomposition of plasmalogen phospholipids. Results are means $\pm$ S.E.M. of four or means of two (where no bars are given) independent experiments.

phase. This radioactivity most probably results from fragments generated by oxidative decomposition of the ^3H -labelled arachidonic acid.

When 16:0/[^3H]20:4-PC-containing micelles were oxidized together with 4–25 nmol of brain lyso-P-PE, the loss of [^3H]PC was dose-dependently prevented (Table 2). Concomitantly, the amount of ^3H in the aqueous phase decreased. With the other plasmalogens tested, oxidative degradation of the labelled diacyl phospholipid was also considerably reduced. Monoacyl and diacyl phospholipids barely affected degradation of 16:0/[^3H]20:4-PC (Table 2).

The amount of 16:0/18:2-PC initially decreased at a rate of 14.1 nmol/h upon treatment with 10 mM AAPH, as determined chemically (Figure 5, upper panel). On addition of 25 nmol of plasmalogens, the oxidative reduction of the PC species was nearly completely inhibited within the first 60 min. After 180 min, the oxidative loss of the diacyl phospholipid was decreased by 51–63% by the plasmalogens, but was not affected by the polyunsaturated diacyl phospholipid soy bean PE. The amounts of plasmalogen phospholipids decreased at rates of 7.4 nmol/h (brain lyso-P-PE), 7.6 nmol/h (brain P-PE) and 8.0 nmol/h (erythrocyte P-PE) between 0 and 180 min (Figure 5, lower panel).

Figure 6 Kinetics of the effect of AAPH on peak amplitudes of enol ether and intrachain double bond methine ^1H of mixtures containing 16:0/20:4-PC plus plasmalogen phospholipids, as detected by ^1H -NMR

The chemical shifts of the enol ether methine ^1H and of intrachain methine protons were identified at 6.0 p.p.m. and 5.4 p.p.m. respectively. Portions of 320 nmol of 16:0/20:4-PC without or with 80 nmol of brain lyso-P-PE (lyso BP-PE), brain P-PE (BP-PE) or erythrocyte P-PE (EP-PE) were enriched in TX-100 micelles (1600 nmol) dispersed in 0.75 ml of $^2\text{H}_2\text{O}$ and treated for up to 180 min with 2 mM AAPH at 37 °C. The values obtained at 0 min were set at 100%. Upper panel: degradation of enol ether methine protons of the alkenyl chains at sn_1 of plasmalogen phospholipids. Lower panel: oxidative degradation of intrachain methine protons. Data are from one representative out of three independent experiments.

AAPH at 2 mM induced a rather rapid decrease in the peak amplitude of the intrachain methine protons of 16:0/20:4-PC within the first 90 min, followed by a slower decline between 90 and 180 min (Figure 6, lower panel). In the presence of the plasmalogens, the initial degradation rate (0–90 min) of the methine ^1H of intrachain double bonds was lowered from 11.3%/h (without plasmalogens) to 4.1–5.9%/h (Figure 6, upper panel). Concomitantly, the peak amplitudes of the enol ether methine ^1H were lowered, with rates of 9.6%/h (brain lyso-P-PE), 12.7%/h (brain P-PE) and 13.7%/h (erythrocyte P-PE) determined for the initial 90 min incubation period. When 0.1 mM EDTA was added to the micellar suspension of 16:0/20:4-PC plus brain lyso-P-PE, the rates of degradation of the enol ether and intrachain methine ^1H were 11.6%/h and 3.7%/h respectively.

In the presence of plasmalogens, the initial degradation rate (0–90 min) of the methine ^1H of intrachain double bonds of soy bean PE was reduced from 13.3%/h (without plasmalogens) to 8.2–10.0%/h (Figure 7, lower panel). In the same time period, the peaks of the methine protons of enol ether double bonds

Figure 7 Kinetics of the effect of AAPH on peak amplitudes of enol ether and intrachain double bond methine ^1H of mixtures containing either soy bean PE or 16:0/18:2-PC plus plasmalogen phospholipids, as detected by ^1H -NMR

Portions of 320 nmol of either soy bean PE (SB-PE) or 16:0/18:2-PC without or with 80 nmol of plasmalogen phospholipids (lyso BP-PE, brain lyso-P-PE; BP-PE, brain P-PE) were enriched in TX-100 micelles (1600 nmol) dispersed in 0.75 ml of $^2\text{H}_2\text{O}$ and treated for up to 180 min with 2 mM AAPH at 37 °C. The values obtained at 0 min were set at 100%. Upper panel: degradation of enol ether methine protons of the alkenyl chains at sn_1 of plasmalogen phospholipids. Lower panel: oxidative degradation of intrachain methine protons. Data are from one representative out of three independent experiments.

decreased at rates of 10.4%/h (brain lyso-P-PE) and 11.3%/h (brain P-PE) (Figure 7, upper panel).

With 2 mM AAPH, the signal of the methine ^1H of intrachain double bonds of 16:0/18:2-PC disappeared at a rate of 9.6%/h in the first 90 min of incubation, with a slower decrease being observed thereafter (Figure 7, lower panel). In the presence of brain lyso-P-PE, the amplitude of this peak was barely lowered (degradation rate of 1.9%/h). After 180 min, the oxidative degradation of the fatty acid was still prevented by 74%. The concomitant decrease in the signal stemming from the enol ether methine protons of brain lyso-P-PE occurred at a rate of 9.8%/h (Figure 7, upper panel; 0–90 min).

DISCUSSION

Plasmalogen phospholipids containing no (brain lyso-P-PE) or, on average, 2.2 (brain P-PE) and 3.7 (erythrocyte P-PE) double bonds in the acyl chains at sn_2 were incorporated into TX-100 micelles and oxidized using the azo initiator AAPH. The early phase of oxidative degradation was evaluated by ^1H -NMR monitoring of the disappearance of signals arising from the (α -vinyl) methine proton of the plasmalogen-specific enol ether

double bond (at sn_1) as well as from the olefinic methine protons of intrachain double bonds (mostly at sn_2). Oxidative degradation was also determined by measuring the decrease in oxidizable substrates by TLC and subsequent phosphate determination, a procedure that reveals extensive decomposition of the hydrocarbon chains of phospholipids occurring in later phases of the oxidation process.

At a molar ratio of polyunsaturated diacyl phospholipids to plasmalogen phospholipids of 4:1, the initial degradation rate of the ester phospholipids was decreased by 43–100% (Figures 3, 5–7). This was apparent even in mixtures in which the ratio of enol ether to intrachain double bonds was 1:40 (100 nmol of 16:0/20:4-PC plus 25 nmol of erythrocyte P-PE). The oxidative degradation of ester phospholipids was decreased to a comparable degree by the three types of plasmalogens. Accordingly, the capacity of plasmalogen phospholipids to decrease oxidation of polyunsaturated ester phospholipids is apparently not markedly influenced by the presence of fatty acids at sn_2 within the plasmalogen molecules themselves. Therefore interactions of peroxy radicals (generated from the decomposition of AAPH) with the enol ether double bond are suggested to be mostly responsible for this behaviour of plasmalogens.

The rates of oxidative degradation of brain lyso-P-PE, brain P-PE and erythrocyte P-PE, as analysed by chemical determinations, were relatively similar (Figure 1). Since brain lyso-P-PE lacks the intrachain double bonds at sn_2 , these data most probably indicate that the oxidative degradation of the alkenyl chain is the major determinant of the sensitivity of brain P-PE and erythrocyte P-PE to attack by peroxy radicals. However, by means of ^1H -NMR spectroscopy, some differences in the degradation rates of the three types of plasmalogens were observed. The peak of the enol ether methine proton decreased more rapidly with erythrocyte P-PE compared with brain P-PE, and the rate of decrease was lowest with brain lyso-P-PE (Figures 2, 6 and 7). These rather subtle differences were most probably not found using chemical determinations because gross changes in phospholipid structure are required in order to detect oxidative degradation by TLC (see above).

Essentially similar findings have been observed previously during heat-induced oxidation of 1-alkenyl-2,3-diacylglycerols [26]. Probably, products of fatty acid oxidation such as, e.g., peroxy radicals are formed by treatment of erythrocyte P-PE and brain P-PE with AAPH which may interact with the enol ether double bond, favouring its decomposition. In line with this hypothesis, it has been proposed that the enol ether double bond of plasmalogens may react with peroxy radicals (generated by oxidative decomposition of polyunsaturated fatty acids) to yield enol ether epoxides [24]. Accordingly, the ability of plasmalogens to prevent the oxidative damage of intrachain double bonds may be related in part to interactions of the enol ether double bond with products of polyunsaturated fatty acid degradation.

According to the results of the present study, it is rather unlikely that the degradation products of enol ether oxidation will promote the decomposition of intrachain double bonds of fatty acids. This can be deduced in particular from the results of Table 2 and Figure 4, which indicate that the loss of oxidizable substrates (16:0/[^3H]20:4-PC and its unlabelled analogue) is dose-dependently prevented by the plasmalogen phospholipids. It is unlikely that transition metal ions contributed significantly to the observed effects of AAPH on the decomposition of plasmalogen and diacyl phospholipids, as the degradation rates determined in micelles dispersed in double-distilled water were rather similar to those in micelles dispersed in the Hepes buffer (see the Results section). Furthermore, the values for the percentage degradation of 16:0/[^3H]20:4-PC (experiments

performed using micelles dispersed in double-distilled water; Table 2) were comparable with those of the unlabelled analogue (micelles dispersed in the Hepes buffer; Figure 3).

The time-dependent decreases in the amounts of brain lyso-P-PE, brain P-PE and erythrocyte P-PE, as induced by AAPH, proceeded only slightly faster than the oxidative degradation of the diacyl phospholipids 16:0/20:4-PC and 16:0/18:2-PC (compare Figure 1 with Figures 3 and 5 and Table 2). This may be interpreted to indicate that the sensitivity of plasmalogen phospholipids to attack by peroxy radicals is not considerably higher than that of polyunsaturated ester phospholipids. Interestingly, however, at 4-fold higher molar ratios of diacyl phospholipids to plasmalogen phospholipids, the degradation of the former molecules was considerably decreased. Among the factors responsible for this effect, the proposed interaction of plasmalogens with degradation products of fatty acid oxidation (see above) may be particularly relevant, as interactions of these products with the enol ether might interrupt the radical chain which otherwise would result in further decomposition of intrachain double bonds. Nevertheless, other (unknown) factors may also play a role. Clearly, further work is needed to clarify the mechanisms responsible for the inhibitory effect of plasmalogens on the oxidation of polyunsaturated fatty acids.

In summary, ^1H -NMR spectroscopy provides a useful tool to analyse *in vitro* the kinetics of the early phase of oxidative degradation of enol ether and intrachain double bonds under on-line conditions. In mixtures of polyunsaturated diacyl phospholipids and plasmalogens (4:1, mol/mol), the oxidative decomposition of the intrachain double bonds of ester phospholipids is considerably delayed, while the enol ether double bond is rapidly degraded. This is especially evident at lower rates of peroxy radical generation and with ester phospholipids containing linoleic acid which (together with arachidonic acid) is the predominant polyunsaturated fatty acid in most biological systems. The results are in agreement with the view that plasmalogen phospholipids act as scavengers of peroxy radicals in lipid environments.

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