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Sphingosine induces the aggregation of imine-containing peroxidized vesicles

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ABSTRACT

Lipid peroxidation plays a central role in the pathogenesis of many diseases like atherosclerosis and multiple sclerosis. We have analyzed the interaction of sphingosine with peroxidized bilayers in model membranes. Cu^{2+} induced peroxidation was checked following UV absorbance at 245 nm, and also using the novel Avanti snoopers®. Mass spectrometry confirms the oxidation of phospholipid unsaturated chains. Our results show that sphingosine causes aggregation of Cu^{2+}-peroxidized vesicles. We observed that aggregation is facilitated by the presence of negatively-charged phospholipids in the membrane, and inhibited by anti-oxidants e.g. BHT. Interestingly, long-chain alkylamines (C18, C16) but not their short-chain analogues (C10, C6, C1) can substitute sphingosine as promoters of vesicle aggregation. Furthermore, sphinganine but not sphingosine-1-phosphate can mimic this effect. Formation of imines in the membrane upon peroxidation was detected by ^{1}H-NMR and it appeared to be necessary for the aggregation effect. ^{31}P-NMR spectroscopy reveals that sphingosine facilitates formation of non-lamellar phase in parallel with vesicle aggregation. The data might suggest a role for sphingosine in the pathogenesis of atherosclerosis.

1. Introduction

Peroxidation of membrane lipids consecutive to oxidative stress in cells has been shown to affect the bilayer biophysical properties, facilitating phospholipid flip-flop or promoting phase separation among other effects [1–5]. Oxidized phospholipids are known to acquire new biological activities not present in their unoxidized precursors, which may modify patho/physiological processes such as multiple sclerosis and atherosclerotic lesions [6,7]. Atherosclerosis is the most common cause for cardiovascular disease and is characterized by inflammation, cell death, fibrosis, and lipid and macrophage accumulation, cholesterol, glycerophospholipids and sphingolipids being commonly found in the lesions [8,9].

Although the overall effect of sphingolipids in the atherosclerotic process is not clear, it is well established that e.g. inhibition of sphingolipid synthesis can reduce circulating VLDL in hamsters and decrease plasma triglycerides that represent a risk factor for cardiovascular disease [10]. Furthermore, sphingosine-1-phosphate increases continuously with atherosclerotic damage and correlates inversely with HDL levels [11,12]. Less attention has been paid to molecules such as sphingosine and sphinganine whose concentrations in serum and plasma are relatively low, probably because they are being converted to sphingosine-1-phosphate and sphinganine-1-phosphate by blood cells [13]. However it has been reported that oxidized LDL increases the activities of both acidic and alkaline ceramidases in smooth muscle cells and elevate cellular sphingosine as well as S1P [14]. Moreover sphingosine is known to influence membrane biophysical properties, increasing the permeability of model and cell membranes [15] and modifying the thermotropic behaviour of lipids among other effects [1,15–19].

Since it is clear that both lipid peroxidation [20] and sphingolipids with membrane-perturbing effects are present in atherosclerotic lesions, it is intriguing whether this pathological process will also be mediated via changes in membrane properties besides the widely studied protein signaling pathways. The purpose of this study was to analyze the possible differences in the interaction of sphingosine with non-oxidized and peroxidized bilayers using model membrane systems, which may help us to understand issues that are more difficult to study *in vivo*. Vesicles composed of different lipid mixtures have been tested, but most assays have been performed with SM:PE:Ch (2:1:1,

Abbreviations: DMPC, (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine); ^{1}H-NMR, (proton nuclear magnetic resonance); LDL, (low density lipoprotein); Liss Rho PE, (Lissamine Rhodamine B phosphatidyl ethanolamine); PAPC, (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine); PA, (phosphatidic acid); PC, (phosphatidyl choline); PE, (phosphatidyl ethanolamine); PI, (phosphatidyl inositol); ^{31}P-NMR, (phosphorous 31 Nuclear Magnetic Resonance); SM, (sphingomyelin); Top Fluor Cer1P, (Top Fluor ceramides-1-phosphate); VLDL, (very low density lipoprotein)

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mol ratio), largely because of the previous experience of this laboratory with that mixture. Our results indicate that sphingosine induces the aggregation of peroxidized lipid vesicles and that this effect may be mediated by the formation of imines or Schiff bases detected by ^{1}H-NMR, as well as by the ability of sphingosine to facilitate the formation of non-lamellar phases in peroxidized bilayers. Since the aggregation of oxidized vesicles can be considered as a model for oxidized lipoprotein aggregation in atherosclerotic lesions [21,22], the finding that sphingosine induces vesicle aggregation may point to this lipid as a novel component in the pathogenesis of atherosclerosis.

2. Materials and methods

2.1. Materials

Sphingosine, sphingomyelin, egg PA, liver PI, egg PC, PAPC, DMPC, TopFluor Cer1P, LissRho PE, E06 Mouse Monoclonal Antibody (IgM) (330001) and cholesterol were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Egg PE (22% C16:0, 37.4% C18:0, 29.4% C18:1, 11.2% C18:2) was purchased from Lipid Products (South Nutfied, UK). Stearylamine, hexadecylamine, dodecylamine, hexylamine, methylamine and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (47168) were from Sigma (Madrid, Spain). Copper dichloride dihydrate ($CuCl_2 \times 2H_2O$) (102733) was from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Vesicle preparation

LUV of diameters 100–150 nm were prepared by the extrusion method [23] using Nuclepore filters of 0.1 μm pore diameter, at a temperature above the transition temperature of the mixtures (40 °C), in 10 mM HEPES, 150 mM NaCl, pH 7.4. For multilamellar (MLV) liposome preparation the lipids were dissolved in chloroform:methanol (2:1) and mixed in the required proportions, and the solvent was evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by leaving the samples under high vacuum for at least 2 h. The samples were hydrated in 10 mM HEPES, 150 mM NaCl, pH 7.4 helping dispersion by stirring with a glass rod. The final phospholipid concentration of both LUV and MLV was measured as lipid phosphorous [24].

2.2.2. Lipid peroxidation assay

LUV of appropriate composition, usually SM:PE:Ch (2:1:1, mol ratio), with or without either 5% PA or 5% PI, were incubated at 37 °C after addition of $CuCl_2$ (10 μM) at time zero. Copper-induced oxidation was checked at 245 nm every hour according to Schnitzer et al. [25], 1 mg/mL lipid as a liposome preparation being added to 3 mL absolute ethanol [26]. Measurements were carried out in a Uvikon 922 spectrophotometer (Kontron instruments, Groß-Zimmern, Germany), using quartz cuvettes with absolute ethanol in the reference cell.

2.2.3. Snooper testing for the detection of lipid peroxidation

Avanti snoopers® consist of a solid support on which lipids can be spotted. In our case snoopers were prepared according to the layout in Fig. 3 and containing 0.64 nmol, 1.28 nmol, or 1.92 nmol LUV composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA. Snooper strips were blocked with 3% BSA (fatty acid free) in TBS (0.8% NaCl, 20 mM Tris–HCl pH 7.4). The blocked membranes were then probed with E06 (200 ng/mL) in TBS containing 1% BSA for 1 h at room temperature. After washing the membranes 3 times with TBS the bound antibody was detected with goat anti-mouse IgM conjugated to HRP (Southern Biotech, Birmingham, AL, USA) at a 1:10,000 dilution in TBS containing 1% BSA for 1 h at room temperature. After washing, bound HRP was visualized on an X-ray film with an enhanced chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA). Additional lipid snoopers were also stained with molybdenum blue, which forms a complex with phosphorous and can be used to verify the presence of phosphorous containing lipids. Fluorescent reference lipids were used to enable the user to identify the orientation of the strip. As a positive control, Avanti Lipid Snoopers® containing 1.28 nmol of PAPC were prepared and allowed to air oxidize for 72 h, and probed with E06 in order to confirm the fidelity of the antibody.

2.2.4. Vesicle aggregation

Liposome aggregation was induced by addition of sphingosine or other alkylamines to peroxidized vesicles. At prefixed time intervals after Cu^{2+} addition aliquots were removed from the vesicle suspension undergoing peroxidation. Aggregation was assayed as an increase in turbidity (absorbance at 400 nm) measured in a Uvikon 922 (Kontron instruments, Groß-Zimmern, Germany) spectrophotometer.

2.2.5. Stopped-flow kinetics

The rates of aggregation kinetics at increasing sphingosine concentrations were determined using a stopped-flow spectrophotometer SFM-3 (BioLogic, France). 0.6 mM peroxidized LUV composed of SM:PE:Ch (2:1:1) + 5% PA were mixed each time with sphingosine solutions of different concentrations in 1:1 volume proportions to a 0.3 mM final LUV concentration.

2.2.6. Nuclear magnetic resonance

60 mM lipid in the form of MLV was incubated with 10 μM $CuCl_2$ at 37 °C for 1 24 h in order to undergo peroxidation and transferred to 5 mm NMR tubes. Data acquisition was performed in a Bruker AV500 spectrometer (Rheinstetten, Germany) operating at 202.45 MHz for ^{31}P, and 500 MHz for protons, with a 5 mm BBI probe with gradients in the *Z*-axis, at increasing temperatures in the case of ^{31}P. The data were recorded and processed with the software TOPSPIN 1.3 (Bruker, Austria).

2.2.7. Mass spectrometry

Samples were lyophilized then reconstituted with 500 μL 65:35:8 chloroform:methanol:water using a 1 mL Hamilton syringe. This approximately 5 mM solution was slightly cloudy. Four drops of methanol + 1 mM ammonium acetate were added and the sample solutions were then clear. The samples were then diluted 1:5 with methanol + 1 mM ammonium acetate such that the concentration was approximately 1 mM. "Without column" scans were performed by flow infusion via an autosampler using an Agilent 1100 Series HPLC and an ABSciex 4000 QTrap mass spectrometer. Injection volume = 5 μL. "With column" scans were performed using a Waters Acquity UPLC and an ABSciex QTrap 5500. Injection volume = 1 μL. The column was an Agilent XDB-C8 1.8 μm 4.6 × 50 mm (S.N. = USHAN01858). The mobile phases were A = 70:30 methanol:water + 5 mM ammonium acetate and B = methanol + 5 mM ammonium acetate. Flow rate = 1.0 mL/min.

3. Results

3.1. Sphingosine-induced vesicle aggregation during copper-induced peroxidation

Peroxidation of LUV consisting of SM:PE:Ch (2:1:1, mol ratio) + 5% PA or PI was followed as UV absorbance at 245 nm and at pre-fixed times aliquots of the sample were taken for the aggregation measurements. Peroxidation increased at a fast rate for 2–3 h, and then it went on more slowly for a long time (Fig. 1 A). The presence of negatively-charged lipids did not modify markedly the rate or extent of peroxidation. The peroxidation process was paralleled by vesicle aggregation in the presence of sphingosine. Aggregation was in this case facilitated by the presence of negatively charged phospholipids in the membrane (Fig. 1 B). This is probably due to electrostatic forces that facilitate the interaction between sphingosine and negatively charged bilayers. Other compositions were tried including vesicles composed of PC, PC:

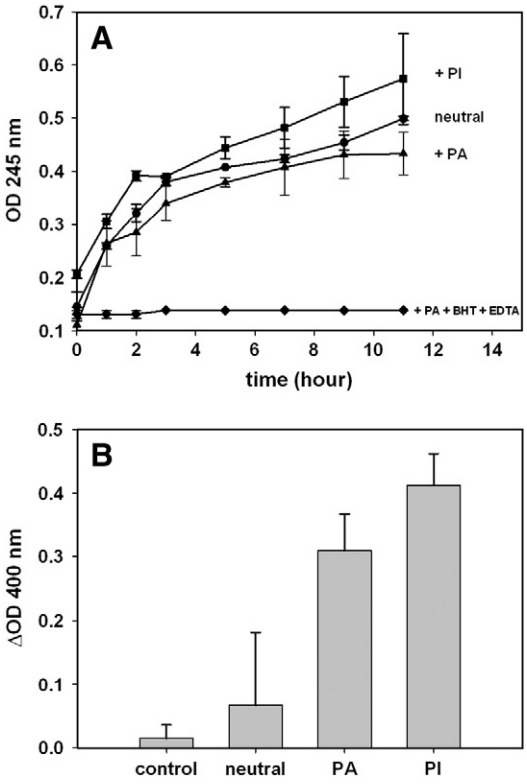

Fig. 1. Copper-induced vesicle peroxidation and sphingosine-induced vesicle aggregation. LUVs composed of SM:PE:Ch (2:1:1 mol ratio) ± 5 mol.% PA or PI. (A) Copper-induced peroxidation. 1 mg/mL of lipid was dissolved in absolute ethanol for the measurements of OD 245 nm. (B) Sphingosine-induced vesicle aggregation after 3 h of copper-induced oxidation. 0.3 mM of LUV was prepared and 20% sphingosine was added. Control experiments with EDTA (chelating agent) (200 μM) and BHT (antioxidant) (50 μM) are also shown in both cases.

PE (2:1, mol ratio) and PC:PE:Ch (2:1:1, mol ratio). In all cases lipid peroxidation was detected following the addition of $CuCl_2$ and sphingosine or long chain amines (see below) were able to induce vesicle aggregation, although to a lesser extent than with the SM:PE:Ch mixture (data not shown). Pure egg PC bilayers were particularly resistant to aggregation. Moreover the fact that vesicle aggregation can be inhibited by the antioxidant BHT confirms that this effect is a direct consequence of membrane peroxidation (Fig. 1).

To study in further detail this aggregation process the time course of aggregation of LUV composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA was recorded at increasing sphingosine concentrations. A stopped-flow spectrophotometer was used to study aggregation from its initial steps. As seen in Fig. 2 A, no aggregation was detected in the non-oxidized bilayer. However, when the membrane was peroxidized, sphingosine induced an increase in turbidity (A_{400}) in a dose-dependent manner (Fig. 2 B). As shown in Fig. 2 C, aggregation rates were higher as sphingosine concentration was increased, showing that the process was also dose-dependent for sphingosine.

3.2. Validation of the capacity of E06 antibody to recognize peroxidized liposomes: a novel method to test lipid peroxidation in membranes

Avanti E06 antibody recognizes the phosphocholine group of oxidized but not of non-oxidized phospholipids [27]. As seen in Fig. 3 A, a signal was obtained specifically for the oxidized sample, suggesting that it is possible to probe peroxidation in our lipid system using the Avanti snoopers. Different snoopers were stained with molybdenum blue, which forms a complex with phosphorous and can be used to detect the presence of phosphorous-containing lipids. Fig. 3 B shows the presence of lipids in all the samples, therefore the fact that a chemiluminescent signal was not detected for the non-oxidized samples probed with E06 is not the result of a differential affinity of these samples for the membrane, but rather of the specificity of the antibody to its substrate. Finally, snoopers containing oxidized PAPC were probed

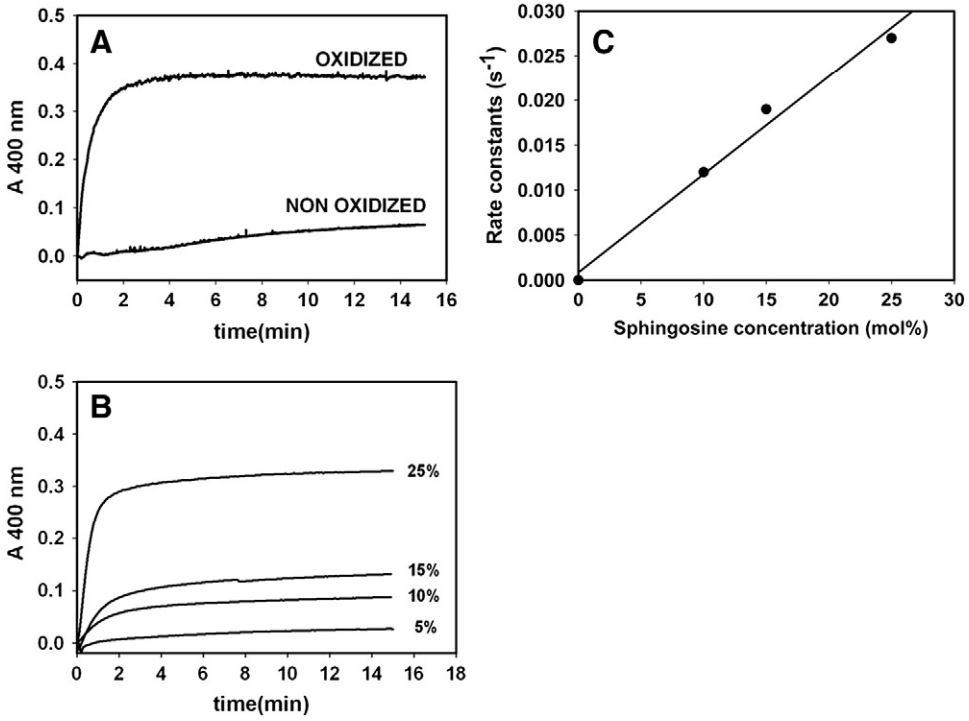

Fig. 2. Time course of sphingosine-induced aggregation. LUVs were composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA. (A) Time-course of turbidity measurements of sphingosine-induced vesicle aggregation. (B) Turbidity measurements of sphingosine-induced vesicle aggregation. Different mol.% sphingosine were added at time 0. (C) Rate constants of vesicle aggregation kinetics, induced by different sphingosine concentrations. Values were fitted to a linear polynomial equation ($r^2 = 0.986$).

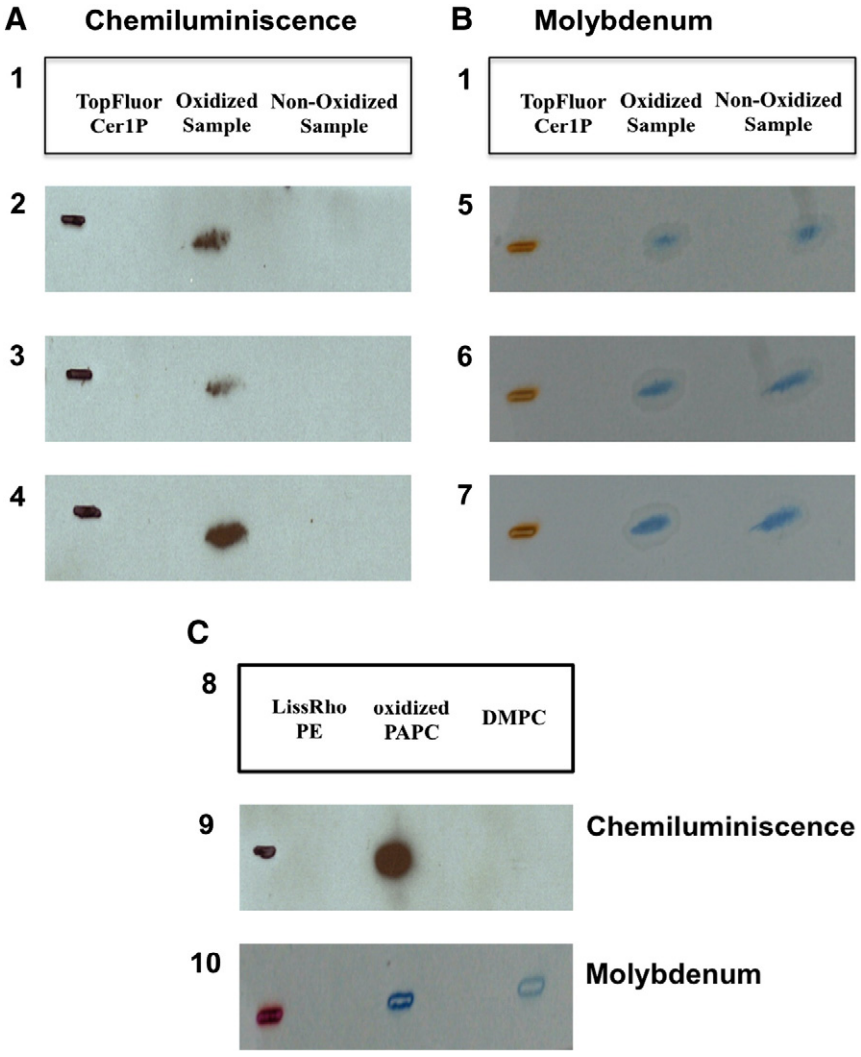

Fig. 3. Snooper testing of lipid peroxidation. (A) Chemiluminescent detection of lipid peroxidation. Avanti® snoopers were prepared according to the layout in the figure containing (1) 0.64, (2) 1.28, or (3) 1.92 nmol of LUV composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA. (B) Molybdenum staining to prove phospholipid presence. Molybdenum blue forms a complex with phosphorous containing lipids. (5) 0.64, (6) 1.28, or (7) 1.92 nmol of LUV composed of SM:PE:Ch (2:1:1, mol ratio) + 5 mol.% PA. (C) Positive control. Avanti® snoopers were prepared according to the layout in the figure containing 1.28 nmol of PAPC, which was allowed to air-oxidize for 72 h. (9) Chemiluminescent detection, (10) molybdenum staining.

simultaneously with the snoopers containing the non-oxidable DMPC (Fig. 3 C) in order to confirm the specificity of the antibody and to serve as a positive control.

3.3. Other alkylamines as promoters of vesicle aggregation

In order to find out which was the sphingosine functional group responsible for the promotion of vesicle aggregation, chain-length dependent aggregation studies were carried out. For this purpose, different chain length amines were used and compared with the effect of the sphingosine. Vesicles were incubated in the presence of 10 μM $CuCl_2$ at 37 °C and aliquots were taken at pre-established time points for turbidity assays. The results are shown in Fig. 4. Long chain alkylamines (C18, C16) can substitute sphingosine as promoters of vesicle aggregation. The effect of stearylamine (C18) was actually more pronounced than that of sphingosine (Fig. 4). However, the chain length of short alkylamines (C10, C6, C1) appears to be insufficient to promote vesicle aggregation, probably because it is not enough to facilitate a proper insertion of the molecule in the bilayer. Thus besides the amino group, a long alkyl chain appears to be required for sphingosine to exert its vesicle aggregation effect.

Furthermore the head group of sphingosine appears to be necessary for this aggregation to occur since its saturated analogue sphinganine induces aggregation while sphingosine-1-phosphate does not (data not shown). Perhaps the net positive charge of sphingosine is important in this respect.

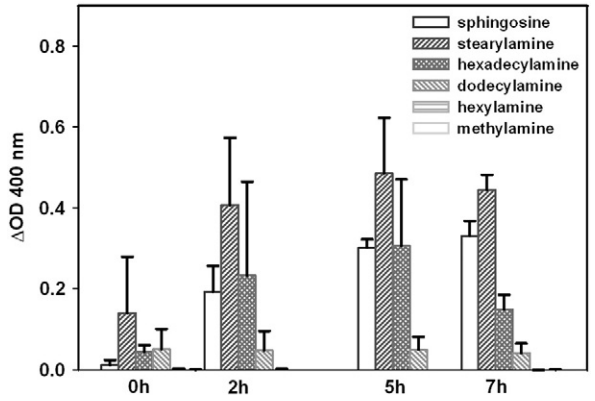

Fig. 4. Other alkylamines as promoters of vesicle aggregation. Sphingosine and other alkylamine-induced aggregation of vesicles composed of SM:PE:Ch (2:1:1) + 5 mol.% PA during their oxidation. At time zero $CuCl_2$ (10 μM) was added.

3.4. Effect of sphingosine on the phase behaviour of peroxidized vesicles

It had been shown earlier that peroxidation of membrane lipids modified their structures, which in turn might affect membrane biophysical properties, including the formation of non-lamellar structures [28]. ^{31}P-NMR is a useful tool in the detection of non-lamellar phases. Experiments were performed using multilamellar vesicles and increasing temperatures, in order to facilitate the transition from lamellar to non-lamellar structures. As seen in Fig. 5, an isotropic signal starts to appear above 65 °C in both cases, vesicles with and without sphingosine. However in the peroxidized vesicles composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA only a weak signal can be detected (Fig. 5 A), while in the vesicles containing sphingosine the signal is larger, becoming predominant at 85 °C. The isotropic signal could indicate the presence of a non-lamellar phase. (Note that the relatively poor signal-to-noise ratio of the spectra containing sphingosine is due to sample inhomogeneities due to the tendency of these vesicles to aggregate and precipitate). The isotropic signal observed in the presence of sphingosine is not due to the presence of micelles or small vesicles, because no significant phospholipid solubilization could be observed under our conditions.

3.5. Detection of imines upon copper-induced peroxidation

^{1}H-NMR has been used to detect the presence of imines in peroxidized vesicles composed of SM:PE:Ch (2:1:1, mol ratio) + 5% PA based on previous published data [29]. A signal assigned to imine protons was detected in these vesicles before and after the addition of 20 mol.% of sphingosine (Fig. 6), while no imine formation was found in MLV with compositions (e.g. pure egg PC) in which sphingosine did not induce vesicle aggregation (data not shown), showing a clear relationship between lipid peroxidation, imine formation in the membrane and sphingosine-induced aggregation.

3.6. Mass spectrometry analysis

The PE component of our vesicles is the likely candidate to undergo formation of a Schiff base. In order to monitor formation of a simple Schiff base, we began by looking for a shift of +13 amu. We attempted this by scanning each of the samples (non-oxidized, oxidized, and oxidized + 20% sphingosine) in positive (+Q1) ion mode. This small shift is extremely difficult to monitor and more difficult to interpret in the mass spectra of a natural lipid or a mixture of natural lipids. This idea was reflected in the collected spectra, as we were unable to visualize any distinct differences in these initial scans. In further attempting to identify differences within the samples, a column was used to separate the lipid classes prior to looking for a neutral loss of 141 (+NL141), which is characteristic of PE species. As shown in pages 19–21 of the Supplementary Material 1, notable differences in chromatography of the +NL141 scans were seen between the three samples reflecting alterations of the PE species. Next, a precursor scan of 184 (+Prec 184)

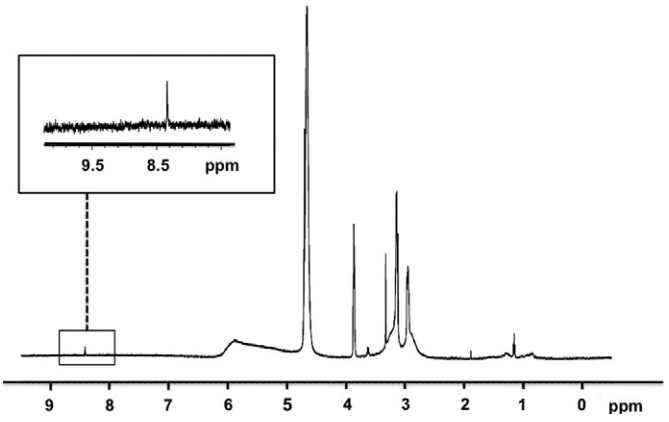

Fig. 6. ^{1}H-RMN of vesicles composed of SM:PE:Ch (2:1:1, mol ratio) + 5 mol.% PA after copper-induced peroxidation showing the detection of imines in the sample.

was performed on the samples in order to look for changes in the sphingomyelin species, although it is unlikely that the tertiary amine would undergo a Schiff base formation. As shown on pages 16–18 of the attached pdf, the differences in chromatography of the +Prec 184 scans, reflecting changes in sphingomyelin species, were not very pronounced between the three samples, suggesting that it is the PE species which is most affected by the copper chloride oxidation. In an attempt to identify aldehyde-modified PE species formed as a result of oxidation, neutral loss scans of 168 (PE + 28) and 182 (PE + 42) were performed. These were chosen because they were the most abundant aldehyde modifications resulting from copper oxidation in the study by Guo et al. [30]. Unfortunately, we were unable to see any peaks above noise in these samples. However, in more closely examining the +NL141 scans (pages 19–21 of the Supplementary Material 1), the most pronounced differences occurred in the *m/z* range of 450–700 reflecting the possibility of specific oxidized acyl chains. In order to identify specific acyl chains undergoing oxidative changes, precursor scans of 283, 281, 279, 255, and 303, which correspond to C18:0, C18:1, C18:2, C16:0, and C20:4 respectively, were performed. These scans comprise pages 1–15 of the Supplementary Material 1. From these scans, the "List of Peaks" table in the Supplementary Material 2 was created. This file lists each peak seen for each scan, and it identifies whether it was present in the non-oxidized, oxidized, or oxidized + 20% sphingosine sample. Taking this data one step further, multiple reaction monitoring (MRM) transitions were then set up for the peaks present in the oxidized samples but not present in the non-oxidized samples. From these scans, peak intensity was then recorded, "Ox Differing Peaks" in Supplementary Material 2, and can be used to monitor the abundance of putative species. For some of the species, potential structures can be proposed (Supplementary Material 3). Please note that these are suggested structures only, as we have no confirmatory data to back up

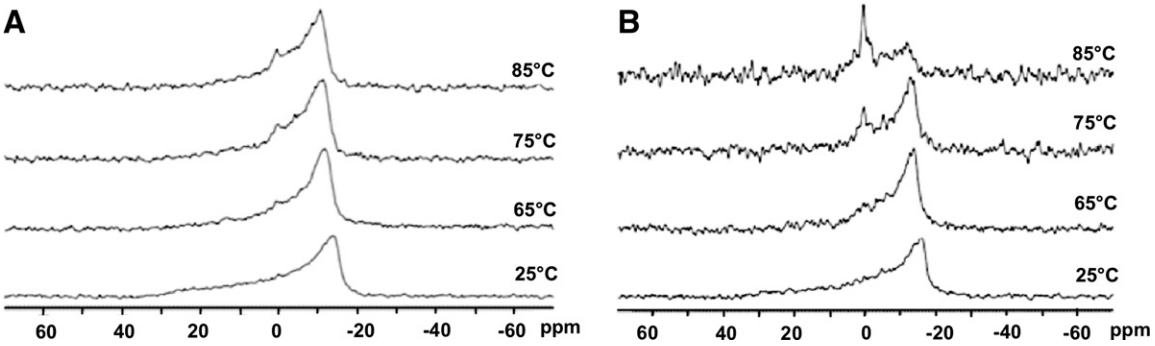

Fig. 5. ^{31}P-NMR experiments of the mixture SM:PE:Ch (2:1:1, mol ratio) + 5 mol.% PA oxidized after the addition of $CuCl_2$ (10 μM) (A) without sphingosine and (B) with 20 mol. % sphingosine.

these suggestions. In summary, although we were not able to identify specific aldehyde-modifications of the PE species, we did find multiple acyl chain differences between the samples. Observation of the actual imine or aldehyde-modified PE species by mass spectroscopy was perhaps made more difficult considering that such species might be forming a complex with the copper used as an inductor of oxidation.

4. Discussion

Lipid peroxidation is a biologically relevant event that takes place in cells under certain conditions such as oxidative stress. The study of this complex process with model membranes is a simplified method to understand the molecular mechanisms involved. In this paper we show that lipid peroxidation can be linked to vesicle, and perhaps lipoprotein, aggregation through sphingosine, the basic building block of sphingolipids. In our study, Cu^{2+}-induced peroxidation of lipid bilayers took place following standard procedures [25,31,32]. From the methodological point of view it is worth mentioning that a simple strip assay for lipid peroxidation has been validated (Fig. 3). Moreover the phenomenon of sphingosine-dependent vesicle aggregation deserves some comment.

Sphingosine induces extensive aggregation of peroxidized vesicles composed of SM:PE:Ch (2:1:1, mol ratio) with or without 5 mol.% of a negatively-charged lipid (PA, PI) (Fig. 1). However aggregation of negatively charged vesicles starts at earlier stages of the peroxidation process. It is likely that aggregation is facilitated by electrostatic interaction between the positively charged sphingosine and the bilayer. This aggregation is peroxidation-dependent since the addition of an antioxidant abolishes sphingosine-induced aggregation. Both the increase in absorbance (extent of aggregation) and the rate of the aggregation reaction are dose-dependent for sphingosine under our conditions (Fig. 2). Note that the negative charge in the bilayer enhances peroxidation by increasing the binding of copper to the interface [22] so that the effect of negatively-charged lipids on the aggregation of peroxidized liposomes is likely to be a complex function of the experimental conditions, including reagent concentration and time of addition. The involvement of sphingosine in peroxidation-dependent aggregation underlines the importance of the control of sphingosine levels in cells, which are usually very low, although under certain circumstances they can be increased [33–35] and its effects are amplified [36,37]. The presence of small amounts of this molecule is known to have a clear impact on the membrane structure [15]. Our ^{31}P-NMR data (Fig. 5) agree with this latter observation since sphingosine is clearly facilitating the formation on non-lamellar intermediates in the peroxidized membrane.

Finding out the functional group responsible for this aggregation effect can be of interest. Sphinganine but not sphingosine-1-phosphate can replace sphingosine (data not shown), probably meaning that the net positive charge of the sphingoid base headgroup is necessary for its action. The increased aggregation observed for vesicles containing negatively-charged lipids also supports a role for the sphingosine positive charge. Furthermore, long chain alkylamines but not their short chain analogues can substitute sphingosine, showing that a long alkyl chain is needed.

Imines or Schiff bases are important chemical compounds arising from the reaction between an aldehyde and an amine, which are found in a variety of biological molecules like retinaldehyde [38]. Different techniques for their detection have been developed, such as Raman spectroscopy [39] or ^{1}H-NMR among others [29]. Lipid peroxidation usually occurs in response to oxidative stress, and a wide diversity of aldehydes is formed when lipid hydroperoxides break down in biological systems [2]. Some of these aldehydes are highly reactive and may react with the amine group of phospholipids in the membrane, specifically phosphatidylethanolamine [40–42], leading to the formation of imines and hence to stable changes in membrane structure. The observation of imine formation in systems in which sphingosine induces aggregation of peroxidized vesicles (Fig. 6) confirms the proposed mechanism of vesicle–vesicle contacts and aggregation.

Our results show that sphingosine does not have the same effect on oxidized SM:PE:Ch (2:1:1, mol ratio) vesicles or on oxidized vesicles with other compositions such as PC, PC: PE (2:1) or PC:PE:Ch (2:1:1, mol ratio). The main difference between these populations of liposomes is that in the one containing SM there is a coexistence of liquid-ordered and liquid-disordered phases, while the PC bilayers are exclusively liquid disordered [15,43]. This can suggest that the presence of membrane microdomains in the bilayer is an important factor for this aggregation effect and that some kind of modifications occurs in LUV composed of SM:PE:Ch after peroxidation that allows sphingosine to differentially exert its aggregation effect.

Peroxidation-induced vesicle aggregation is interesting because it can be used as a model for lipoprotein aggregation under oxidative conditions [44,45]. Aggregation of lipid droplets and LDL is an early step necessary for the generation of atherogenic damage. It has been reported that modifications of the surface of these particles, which are enriched in phospholipids (PC, SM), and in cholesterol, can result in loss of particle stability and affect interaction between them, leading to their aggregation [46]. A possible molecular mechanism for this, as we show in this work, could be the formation of imines upon lipid peroxidation. Hence, this molecular mechanism of action of sphingosine when interacting with imine-containing peroxidized bilayers may be biologically relevant and can help us understand the overall role of sphingolipids in atherosclerosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.04.028.

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