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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: