Published in final edited form as:

Arch Biochem Biophys. 2009 January 1; 481(1): 72–79. doi:10.1016/j.abb.2008.10.005.

Effect of double bond geometry in sphingosine base on the antioxidant function of sphingomyelin

Papasani V. Subbaiah^{1,*}, Debajit Sircar¹, Ravi S. Lankalapalli², and Robert Bittman²

¹ Department of Medicine, University of Illinois at Chicago

² Department of Chemistry and Biochemistry, Queens College of the City University of New York, Flushing, NY 11367

Abstract

We previously showed that sphingomyelin (SM) inhibits peroxidation of phosphatidylcholine (PC) and cholesterol. Since SM uniquely has a trans unsaturation in its sphingosine base, we investigated whether this feature is important for its antioxidant function. Substitution of the natural trans Δ^4 -double bond with a cis double bond (cis-SM), however, increased SM's ability to inhibit Cu²⁺-mediated 16:0–18:2 PC oxidation by up to 8-fold. Dihydro-SM, which lacks the double bond, was equally effective as trans-SM. In contrast to its effect in the sphingosine base, the presence of a cis double bond in the *N*-acyl group of trans-SM was not protective. cis-SM also inhibited the oxidation of cholesterol by FeSO₄/ascorbate more efficiently than the trans isomer. The enhanced protective effect of cis-SM is selective for metal ion-promoted oxidation, and appears to arise from a decrease in the effective concentration of metal ions. These studies show that the trans double bond of SM is not essential for its antioxidant effects.

Supplementary Keywords

Double bond geometry; Sphingomyelin; Antioxidant; Cholesterol oxidation; Phosphatidylcholine oxidation; Liposomes

Introduction

Sphingomyelin (SM) is an abundant phospholipid in the cell membranes and lipoproteins of all vertebrate animals, and is an essential component of membrane rafts, the microdomains of cell membrane that play a critical role in cell signaling and other cellular processes [1,2]. SM is concentrated in the plasma membrane of cells, specifically in the exofacial leaflet that faces the environment. Recent epidemiologic studies show that increased plasma levels of SM are associated with an increase in risk of atherosclerosis [3,4], but the underlying mechanisms are largely unknown. Part of the reason for this is that the normal physiological functions of SM in cells and lipoproteins are not well understood. A well-established role of membrane SM is as a precursor of signaling molecules such as ceramide and sphingosine 1-phosphate. However, since the amount of SM present in the plasma membrane far exceeds the signaling requirements

*Address correspondence to: P.V. Subbaiah, Ph.D., Section of Endocrinology and Metabolism, University of Illinois at Chicago, 1819 West Polk Street, Chicago, IL 60612, Email: psubbaia@uic.edu, Phone: 312-996-8212; FAX: 312-413-0437.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of the cell, it is likely that SM plays other roles in the membrane. Similarly, the physiological function of SM in lipoproteins, where it is the second most abundant phospholipid, is not established. We and others have shown that SM is a physiological modulator of cholesterol esterification [5–7] and of various lipolytic reactions in cell membranes and lipoproteins [8–11], possibly acting as a competitive inhibitor because of its structural similarities to PC [12]. We have also demonstrated that SM inhibits the oxidation of unsaturated PC and cholesterol in lipoproteins and cell membranes [13,14]. However, the possible mechanism for this antioxidant function is not clear. Since SM does not have the properties of a prototypical antioxidant, such as the ability to scavenge free radicals (e.g., peroxy or hydroperoxy radicals), we proposed that it inhibits the propagation of the lipid peroxy radicals by virtue of its oxidation-resistant structure [12–14]. Another mechanism, proposed by Oborina and Yappert [15], is that hydrogen bonding between neighboring SM molecules results in the formation of a strong interfacial barrier that inhibits the penetration of free radicals. Since these mechanisms are not mutually exclusive, it is important to determine the structural features of SM that are essential for its antioxidant function.

A unique structural feature of SM (and other sphingolipids) is the presence of a trans double bond in its sphingosine backbone, in contrast to the other naturally occurring membrane phospholipids which have cis unsaturation in their fatty acyl groups. In sphingolipid biosynthesis, the trans double bond is introduced into the long chain base of ceramide by the stereospecific dihydroceramide desaturase. The lack of this enzyme leads to cell cycle arrest in cultured cells [16] and to growth retardation and death in experimental animals [17]. Therefore, it is likely that the trans double bond in SM has unique functions that are not fulfilled by the more common cis unsaturation found in glycerolipids. As a trans double bond tends to be less reactive chemically than a cis double bond, it may also be less susceptible to free-radical reactions. Unlike the cis double bond found in natural fatty acids which are in the interior of the bilayer, the trans double bond of SM is located at the C4-C5 of its sphingosine backbone, and resides near the interfacial region, in the vicinity of the hydrogen bond forming C3-OH and C2-NH groups. Physicochemical studies revealed that the presence of a double bond in this region of the membrane strengthens hydrogen bonding, increases the dipole moment, and enhances the hydration of the polar region [18] [19]. We have previously shown that the presence of a trans double bond in the fatty acyl groups of PC not only reduces its oxidizability by peroxy radicals, but also inhibits the oxidation of the neighboring cis unsaturated PC molecules [20]. It is, therefore, of interest to determine whether the trans double bond of the sphingosine backbone plays a role in the antioxidant effects of SM. For this purpose, we synthesized an unnatural analog of SM containing a cis double bond at the C4 position of the sphingosine backbone, and determined its effect on PC and cholesterol oxidation induced by Cu²⁺ or by the extensively studied water-soluble peroxy radical generator 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH). Unexpectedly, the results show that the cis double bond at this position is several fold more effective than the trans double bond in the protection of PC and cholesterol against oxidation, especially in the presence of cupric ions

MATERIALS AND METHODS

Materials

Egg SM (which consists of approximately 85% *N*-palmitoyl-SM, 6% *N*-stearoyl-SM), diethylenetriamine pentaacetic acid (DTPA), diphenylpicrylhydrazyl radical (DPPH), and methyl β -cyclodextrin (M β CD) were purchased from Sigma (St. Louis, MO). *N*-Stearoyl-SM was obtained from Matreya (Pleasant Gap, PA). Synthetic PCs (18:1-18:1 PC, and 16:0-18:2 PC) were purchased from Avanti Polar Lipids (Alabaster, AL). AAPH was purchased from Wako Pure Chemical Industries (Richmond, VA), and 4-[¹⁴C]cholesterol (54 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Synthesis of SM analogs

The structures of the SM analogs used here are shown in Chart 1. A detailed description of the synthesis, purification, and characterization of the SM analogs is presented in the Supplement. Briefly, for the synthesis of *cis*-SM, the reaction of (*S*)-Garner aldehyde with the lithium salt of 1-pentadecyne in HMPA/THF (nonchelating conditions) at -78°C provided a mixture of erythro and threo propargylic diastereomers that were separated by flash chromatography; the ratio of D-erythro to L-threo product was $\sim 20:1$ [21,22]. The erythro diastereomer was treated with Amberlyst-15 resin in methanol, affording a *N*-Boc-protected diol. Partial reduction of the triple bond by reaction with hydrogen in the presence of Lindlar catalyst [23] in ethyl acetate for 25 min afforded *cis*-*N*-Boc-D-erythro-sphingosine. The phosphocholine group was incorporated by using ethylene chlorophosphite and bromine at -78°C in CH_2Cl_2 , followed by quaternization using aqueous trimethylamine in $\text{CH}_3\text{CN}/i\text{-PrOH}/\text{CHCl}_3$ (5:5:3, v/v), affording *N*-Boc-D-erythro-4,5-*cis*-sphingosylphosphorylcholine as a white powder. Deprotection of the *N*-Boc group with HCl in aqueous tetrahydrofuran at 70°C provided lyso-4,5-*cis*-SM, which was subjected to *N*-acylation with *p*-nitrophenyl palmitate in dry DMF/ CH_2Cl_2 (5:2, v/v) in the presence of anhydrous potassium carbonate as described previously [24], affording (2*S*,3*R*,4*Z*)-*N*-palmitoyl SM (*cis*-SM). LC/MS analysis of this compound showed the presence of $\sim 10\%$ of dihydro-SM. *trans*-SM containing a $16:1\Delta^4$ *cis* *N*-acyl chain was synthesized by *N*-acylation of D-erythro-lyso-SM with *p*-nitrophenyl (*Z*)-hexadec-4-enoate. *N*-Oleoyl SM was similarly synthesized by *N*-acylation of D-erythro-lyso-SM with *p*-nitrophenyl oleate. L-threo *N*-palmitoyl-SM [(2*S*,3*S*,4*E*)-SM] was prepared by *N*-acylation of L-threo-lyso-SM (Matreya) with *p*-nitrophenyl palmitate. Dihydro-D-erythro-SM was prepared by catalytic hydrogenation of D-erythro-sphingosylphosphorylcholine (Matreya), followed by *N*-acylation with *p*-nitrophenyl palmitate.

Assay of PC peroxidation

Liposomes (large unilamellar vesicles, LUVs) containing 16:0-18:2 PC and various SM analogs (molar ratio of PC:SM, 5:1) were prepared by extrusion through polycarbonate filters. Briefly, chloroform solutions of PC (2 μmol), with or without SM (0.4 μmol), were added to a glass tube, and the solvent was evaporated under nitrogen. The lipids were dissolved in 0.3 ml of ethanol, and the solvent was again evaporated under nitrogen. To the dried lipid film was added 1 ml of Tris buffer (20 mM Tris, pH 7.4), and the suspension was dispersed by vortexing for 1 min. The sample was flushed with nitrogen, incubated in the dark at 40°C for 20 min, and passed through a 0.1 μm Nucleopore membrane (Whatman) 11 times using a Mini-extruder apparatus (Avanti Polar Lipids), while maintaining the temperature of the syringe at 40°C with the help of a heating block. The liposomes were stored under nitrogen at 4°C in the dark, and were used for oxidation studies within 10 days of the preparation. The ratios of PC and SM in the recovered LUVs were estimated by lipid phosphorus analysis [25] after TLC separation of lipids, and were found to be the same as in the starting material. For the oxidation studies, 120 μl of the liposome preparation (240 nmol of PC) was treated with 50 μM CuCl_2 in 10 mM Tris, 150 mM NaCl, pH 7.4, in a final volume of 3.0 ml, in the cuvette of a spectrophotometer. The reactions were carried out at 37°C for up to 600 min in a temperature-controlled spectrophotometer (Shimadzu UV-1601), and the readings at 234 nm (conjugated diene formation) were recorded continuously at 5-min intervals. The rates of oxidation were calculated from the slopes in the linear range, after subtraction of the corresponding blank values (samples incubated in the absence of Cu^{2+}). In some experiments, the oxidation was carried out in presence of the thermolabile free-radical generator AAPH (0.1 mM) instead of CuCl_2 .

Assay of cholesterol oxidation

Liposomes containing 18:1-18:1 PC, SM, and radiolabeled free cholesterol were prepared by the cholate dialysis procedure [26], but in the absence of apoprotein A1. Briefly, chloroform solutions of PC (2 μ mol) and the SM analog (0.4 μ mol, where indicated), and 4-[14 C] cholesterol, 0.2 μ mol) were added to a glass tube, and the solvent was evaporated under nitrogen. The lipids were re-dissolved in 0.5 ml of ethanol, and the solvent was again evaporated under nitrogen. To the dried lipid film, 100 μ l of 725 μ M cholate solution in Tris-NaCl buffer (10 mM Tris, 0.15 M NaCl, pH 7.4) was added, and the suspension was vortexed for 1 min to disperse the lipids. The sample was then flushed with nitrogen, incubated at 37 °C in the dark for 20 min, and dialyzed extensively at 4 °C in the dark (4 changes of 200 volumes of Tris-NaCl buffer, pH 7.4). The volume of the dialyzed sample was adjusted to 1.0 ml with Tris-NaCl buffer, and stored under nitrogen at 4 °C in the dark. The liposomes were used for the oxidation studies within 10 days of preparation.

Liposomes (100 μ l) containing labeled cholesterol were incubated either with 10 mM AAPH or with 0.5 mM FeSO₄/5 mM ascorbate for varying periods of time at 37 °C in a final volume of 0.4 ml, and the reaction was stopped by extraction of the lipids [27]. The total lipid extract was applied to silica gel TLC plates, which were developed with the solvent system of petroleum ether/diethyl ether/acetic acid (60:40:1, v/v). The lipids were visualized by exposure to iodine vapors, and the unoxidized cholesterol spot was identified with the help of a standard run on the same plate. The radioactivity between the cholesterol spot and the origin was taken as that of the oxidation products of cholesterol, and was divided into two bands. The most polar band (origin) was tentatively identified as a mixture of 7 α -hydroxy- and 7 β -hydroxycholesterol, based on the R_f values in the literature [28]. The less polar product (migrating between cholesterol and the origin) was similarly identified as predominantly 7-ketocholesterol. No radioactivity was found above the cholesterol spot. The spots corresponding to oxidized and unoxidized cholesterol were scraped, and their radioactivity was measured in a liquid-scintillation counter. In addition, an aliquot of the aqueous layer after the lipid extraction was counted for radioactivity. Typically, less than 0.2% of the total radioactivity was recovered in the aqueous layer. Of the radioactivity in the total oxidation products, 80–90% was found in the 7-ketocholesterol spot, and the rest was at the origin (7 α -hydroxy- and 7 β -hydroxycholesterol). The oxidation of cholesterol was calculated as the percent of initial 14 C-cholesterol counts found in all of the oxidation products (two bands from TLC and the aqueous layer).

Cholesterol-binding assay

The cholesterol-binding efficiency of LUVs containing various SM analogs was estimated from the equilibrium partition coefficients as measured with the cyclodextrin-cholesterol method described by Niu et al. [29], with a few modifications. The M β CD-cholesterol complex was prepared with 14 C-labeled cholesterol in order to measure the transfer of cholesterol more accurately. LUVs containing 16:0-18:2 PC with or without SM at a 4:1 molar ratio of PC:SM (keeping the total phospholipid concentration constant) were prepared by extrusion. Briefly, solutions of the phospholipids in chloroform were added to a glass tube and the solvent was evaporated under nitrogen. The lipids were redissolved in 160 μ l of cyclohexane containing 50 μ M of butylated hydroxytoluene, the sample was frozen on dry ice, and lyophilized for 4 h to completely remove the solvent. The lipids were then suspended in 10 mM PIPES buffer containing 50 μ M DTPA, pH 7.0. The suspension was extruded through a 0.1 μ m Nucleopore membrane (11 times) at 40 °C as described above. Each LUV suspension (50 μ l) was incubated with 10 μ l of 4-[14 C]cholesterol-M β CD complex (8,000 dpm) and 50 μ l of PIPES buffer for 2 h at 37 °C on a metabolic shaker. After dilution with 100 μ l of 4 M NaCl, the reaction mixture was filtered through a Microcon YM-30 membrane by centrifugation (7000 rpm, 37 °C, 15 min). The filter was washed twice with 200 μ l of 4 M NaCl, each time centrifuging the sample

for 20 min at 7000 rpm to collect the filtrate. The liposomes on the filter were finally collected by washing from the top (no centrifugation) with 800 μ l of 4 M NaCl solution. Aliquots of the liposomes and the filtrate were counted for radioactivity in a liquid-scintillation counter, and the percent of label transferred to the LUVs was calculated. Control samples containing no LUVs were included in each experiment to correct for any non-specific binding of the label to the filter, which was negligible.

Other methods

Binding of Cu^{2+} to SM analogs was estimated from the amount of Cu^{2+} extracted into the lipid phase after incubation of liposomes with CuSO_4 , as described by Hahnel et al. [30]. ^1H NMR spectra were recorded on a Bruker 400-MHz spectrometer. Aliquots of a $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ stock solution in CD_3OD (80 mM) were added to an NMR tube containing SM (1 mg) dissolved in CD_3OD , with shaking. The chemical shifts of the vinylic proton signals are referenced to tetramethylsilane (δ 0.00). The ability of SM analogs to scavenge free radicals was determined by measuring the decline in the absorbance at 517 nm of the diphenylpicrylhydrazyl radical after its incubation with the liposomes, as described by Mathiesen et al. [31]. The PC and SM concentrations following oxidation was determined in some experiments by LC/MS in the positive ionization mode, essentially as described by Ravandi et al. [32], using a normal-phase silica column and a Thermo Finnigan Surveyor MSQ as detector. We employed 17:0-17:0 PC as the internal standard for the measurement of both PC and SM.

RESULTS

SM unsaturation and inhibition of PC oxidation

To determine the effect of unsaturation in the long chain base, we incorporated three SM analogs (dihydro-SM, cis-SM, and trans-SM) into liposomes containing 16:0-18:2 PC at PC:SM molar ratios of 5:1, and determined the oxidation of PC in the presence of 50 μM Cu^{2+} at 37 °C. As shown in Fig. 1, the presence of natural trans-SM (egg SM) increased the lag phase by 30 min and inhibited the rate of PC oxidation by about 20%, based on the linear oxidation rates. However, the incorporation of cis-SM increased the lag phase by 120 min, and inhibited the rate of oxidation by about 90%. The rate of PC oxidation was about 8 times higher in the presence of trans-SM relative to that in liposomes prepared with cis-SM. Dihydro-SM inhibited the oxidation by about 45%. Based on the total conjugated diene concentration at 300 min, the inhibition in the extent of PC oxidation was 31% in the presence of trans-SM, 36% in the presence of dihydro-SM, and 87% in the presence of cis-SM. It may be pointed out that the increased protection of PC by cis-SM does not result from the presence of small amounts of dihydro-SM in the cis-SM preparation, since pure dihydro-SM showed a lower inhibition than cis-SM. Since the amount of PC was the same in all samples, the effects are not due to the dilution of PC.

The effect of the concentration of cis or trans SM in the liposomes on the oxidation of 16:0-18:2 PC is shown in Fig. 2. The inhibition of oxidation was calculated from the decrease in the slopes of A_{234} , relative to PC control with no SM. At all the concentrations tested, cis SM was more inhibitory than trans SM. At 50 mol% the oxidation of PC was inhibited by >90% in presence of cis SM, whereas the inhibition was only about 40% with trans SM.

Increasing the Cu^{2+} concentration by two-fold did not significantly affect the rate of PC oxidation in the presence of cis-SM, but increasing it four-fold (to 200 μM) markedly increased the rate (Fig. 3). The effect of increasing the Cu^{2+} was less noticeable in the presence of trans-SM. The ratio of slopes of PC oxidation (trans-SM/cis-SM) was 7.3 at 50 μM Cu^{2+} , but only 2.7 at 200 μM Cu^{2+} . These data suggest that the difference between the two isomers may result

from the effective concentration of Cu^{2+} , since the differences are reduced by increasing the Cu^{2+} concentration.

When the thermolabile free-radical generator AAPH was used as the oxidizing agent, the protection by cis-SM was less remarkable (Fig. 4). Although the presence of cis-SM in the liposomes induced a significant increase of the lag phase (lag times: 23 min for PC control, 32 min in presence of egg SM, 93 min in presence of cis-SM, and 34 min in presence of dihydro-SM), the rates of oxidation (slopes) in the maximal linear range were similar in the presence of the three analogs of SM, all of which decreased the rate of PC oxidation by about 20%. These results suggest that the inhibitory effect of cis-SM was selective for the Cu^{2+} -mediated oxidation.

Effect of cis bond in the N-acyl group of SM

Unlike the C4 trans double bond of the sphingosine base, the double bonds in the acyl groups of all membrane lipids are located near the middle of the chain, and therefore reside in the interior of the biological membranes. Although the natural SM in biological membranes contains predominantly saturated *N*-acyl groups, monounsaturated *N*-acyl groups are also present [33]. It is, therefore, of interest to investigate whether a cis double bond in the *N*-acyl group of SM has a similar effect as in the long chain base. For this purpose, we synthesized SM analogs containing the natural trans Δ^4 -sphingosine backbone, with either 18:0 or 18:1 (Δ^9 cis) as the *N*-acyl group, and tested their effects on PC oxidation. With *N*-(C18:1 Δ^9 cis)-SM, the lag period (240 min) was increased compared with egg SM (120 min) or *N*-18:0 SM (120 min), but the rate of PC oxidation (after the lag period) was not inhibited (Fig. 5). Not surprisingly, the effect of *N*-18:0 SM was similar to that of egg SM, since >95% of *N*-acyl groups of egg SM are saturated. In order to determine whether the position of the cis double bond in the *N*-acyl group affects the rate of PC oxidation, we also tested the effect of a SM analog containing a *N*-(C16:1 Δ^4 cis) chain, in which the unsaturation is located at an approximately equivalent position as the double bond in the sphingosine backbone relative to the lipid-water interface. However, as shown Fig. 5, this SM analog did not show any protection against PC oxidation, suggesting that the double bond configuration of the sphingosine backbone, and not in the *N*-acyl chain, is important for the inhibition of PC oxidation we observed.

Effect of cis-SM on cholesterol oxidation

Our previous results showed that in addition to PC, SM also inhibits the oxidative degradation of cholesterol [14]. To determine the possible role of the double bond geometry of SM in the protection of cholesterol against oxidation, we prepared 18:1-18:1 PC liposomes containing [4- ^{14}C]-cholesterol (10 mol%), and the cis or trans analog of SM (20 mol%) and studied the degradation of radiolabeled cholesterol in the presence of FeSO_4 -ascorbate (since Cu^{2+} does not oxidize cholesterol appreciably [14]). Since 18:1-18:1 PC is not oxidized under these conditions, the confounding effects of the PC oxidation products on cholesterol oxidation are eliminated. As shown in Fig. 6, cholesterol oxidation was inhibited more strongly by cis-SM than by trans-SM. It should be noted that the extent of cholesterol oxidation is much lower than that of PC oxidation even after prolonged incubation (31 h), and that the differences between the effects of the trans and cis isomers of SM are also less striking compared with the effects on PC oxidation (Fig. 1). In the presence of AAPH (10 mM), however, cis-SM was less effective than trans-SM in inhibition of cholesterol oxidation (Fig. 7), although the differences between the two were not statistically significant.

Effect of double bond geometry on cholesterol binding

It is well established that SM and cholesterol exhibit greater affinity to each other than to other membrane lipids [34]. This interaction contributes to the co-localization of the two lipids in

cell membranes, and to the formation of membrane microdomains or rafts. Since the affinity of SM to cholesterol may play a part in its protection of cholesterol against oxidation, we estimated the cholesterol-binding property of various SM analogs using PC liposomes as acceptors, and the M β CD-labeled cholesterol complex as the donor [35]. The liposomes contained 16:0-18:2 PC and the indicated SM analog (30 mol%). As shown in Fig. 8, all of the SM analogs increased the ability of PC liposomes to extract labeled cholesterol from the M β CD-cholesterol complex. However, natural SM (trans-SM) increased the binding capacity more strongly than cis-SM. Dihydro-SM was slightly more effective than trans-SM, in accordance with the results of Kuikka et al. [35]. Trans-SM containing a C18:0 *N*-acyl group increased the binding marginally compared with egg SM, in which the *N*-acyl chain was composed of 85% C16:0 and 12% C18:0. *L*-threo-SM with a C16:0 amide chain was also marginally more effective than the natural *D*-erythro trans analog, suggesting that the configuration at C-3 of the sphingosine backbone of SM does not play a role in the binding with cholesterol. Since the presence of a 4,5-cis double bond in the long chain base of SM decreases the cholesterol-binding capacity of SM, the protection against cholesterol oxidation in the presence of FeSO₄-ascorbate is not correlated with the affinity of SM to cholesterol.

Chelation of Cu²⁺ by cis SM

Since the inhibition of PC oxidation by cis-SM was greater when Cu²⁺ was the oxidizing agent (compared with AAPH), and since its effect can be partially reversed by increasing the Cu²⁺ concentration, we explored the possibility that cis-SM acts as a Cu²⁺-chelating agent. For this purpose, liposomes containing 16:0-18:2 PC alone, or PC + SM at 2:1 molar ratios, were incubated with Cu²⁺ for 5 min at room temperature, and the Cu²⁺ remaining in the aqueous layer was determined after extraction of the lipids [27]. There was no significant difference between liposomes containing PC alone and those containing either PC + trans-SM or PC + cis-SM with respect to the ability to bind Cu²⁺ under the conditions of the assay. There was also no difference in binding when liposomes containing only SM (cis vs trans) were compared (results not shown).

The interaction of cis and trans isomers of SM with Cu²⁺ was also studied using NMR. When the effect of Cu²⁺ on the chemical shifts of the vinylic protons of cis- and trans-SM was determined by NMR at a SM:Cu²⁺ ratio of 1.0, we found a small downfield shift of the signal in the case of cis-SM (from 5.375 to 5.395 ppm for the proton at C4, and from 5.517 to 5.537 ppm for the proton at C5) but not in the case of trans-SM (results not shown). However, these differences were not large enough to explain the great differences in the antioxidant effects of the two isomers. Furthermore, there was no significant difference in T1 relaxation times of the vinylic protons of cis and trans SM isomers in presence of Cu²⁺. These results appear to rule out chelation of Cu²⁺ as a major contributing factor. We also tested the possibility that cis-SM acts as a free-radical scavenger, using the diphenylpicrylhydrazyl radical (DPPH) assay, as described by Mathiesen et al. [31]. There was no significant scavenging effect by any of the SM analogs (cis SM, trans SM, dihydro SM) under the conditions of assay (results not shown), indicating that the difference between cis-SM and trans-SM is not due to this property.

Cu²⁺-mediated oxidation of PC in liposomes is not accompanied by oxidation of SM

We next investigated the fate of SM during the oxidation reaction by analyzing the total lipid extract by LC/MS before and after Cu²⁺-mediated oxidation of 16:0-18:2 PC. There was no significant change in the concentration of SM during the oxidation reaction, although the amount of PC decreased as expected (Fig. 9.) No degradation products of SM could be detected in the presence of either trans-SM or cis-SM, but the mono and dihydroperoxy derivatives of PC increased during oxidation, as expected (results not shown). These results show that the protective effect of SM is not due to a sparing effect on PC oxidation.

DISCUSSION

Lipid peroxidation has been implicated in the etiology of several diseases, including atherosclerosis, cancer, diabetes, and Alzheimer's [36]. The study of endogenous and diet-derived antioxidants is, therefore, of obvious clinical and therapeutic importance. While the majority of the known antioxidants work through the scavenging of free radicals or breaking the chain reaction, some may act through their membrane-altering properties. For example, the antioxidant effects of several synthetic estrogens, as well as tamoxifen and cholesterol, have been shown to be positively correlated with their ability to decrease membrane fluidity [37, 38]. We previously showed that SM inhibits the peroxidation of unsaturated phospholipids and cholesterol and suggested that it acts as a natural antioxidant [13,14]. Since SM is not structurally similar to the typical antioxidants and does not have the ability to scavenge free radicals, we proposed that its mechanism of action also involves membrane stabilization and inhibition of the propagation of lipid peroxy radicals [12]. Support for such a mechanism stems from the presence of a high concentration of SM in membranes and lipoproteins, and the known ability of SM to decrease membrane fluidity because of its saturated structure. However, SM has several other characteristics that may be important in its antioxidant activity. For example, its ability to form intramolecular and intermolecular hydrogen bonds may be important in inhibiting the penetration of the free radicals, as suggested by Oborina and Yappert [15]. Another unique characteristic of SM is the trans configuration of the double bond in its sphingosine backbone, which is rare among natural membrane lipids. While the presence of a double bond in SM appears to be essential for cell survival [16], the physiological importance of the trans geometry of the double bond in SM has not been investigated. Our earlier studies showed that the presence of a trans double bond in the fatty acyl groups of PC inhibits lipid peroxidation in lipoproteins and cell membranes [20]. Therefore, we studied the possible importance of the trans configuration in the antioxidant function of SM, by employing a synthetic cis analog of SM. The major finding of the study was that replacing the trans double bond in SM with a cis double bond paradoxically resulted in up to an eight-fold increase in its antioxidant activity in the presence of Cu^{2+} as the oxidizing agent. This effect of the cis double bond was less evident when AAPH was used as the oxidizing agent. In contrast to the effect of the cis double bond in the sphingosine backbone, its presence in the *N*-acyl group of SM did not confer an increased antioxidant property, even when the double bond was present at an approximately equivalent position (C4) as in the long chain base. These results suggest that the double bond geometry at the C4 position in the sphingosine base plays an important role in the inhibition of PC oxidation brought about by Cu^{2+} .

The mechanism by which the cis double bond in sphingosine base increases the antioxidant property of SM, especially in metal ion catalyzed reactions, is not clear. One possibility is that cis-SM may act as a metal chelating agent, decreasing the effective concentration of Cu^{2+} . Our NMR studies, as well as the direct binding studies, however, did not show a significant chelation of Cu^{2+} by either cis or trans SM. Another possibility is that it acts as a weak free-radical scavenger, removing the small amounts of pre-formed radicals in the sample. Since Cu^{2+} -mediated oxidation is dependent upon pre-formed radicals [39], it would be inhibited by cis-SM, whereas AAPH-mediated oxidation would not be inhibited because it generates free radicals continuously by thermal decomposition. Our studies on DPPH radical scavenging, however, did not show any scavenging effect for either cis or trans SM. It is of interest to note that ethanolamine plasmalogen also inhibits the oxidation of PC when the oxidation is carried out in presence of transition metal ions but not when AAPH is the oxidizing agent [40]. In this case the chelation of the metal ion was proposed as the possible mechanism. However, the later studies of Sindelar et al. [41] did not support this mechanism, but instead suggested that the plasmalogens interfere with the propagation of the peroxidation reaction.

The geometry of the double bond in the sphingosine moiety of SM does not affect all of the properties of SM in the same manner. In addition to the inhibition of lipid peroxidation, SM is known to inhibit the activities of several lipolytic enzymes that utilize PC as substrate, possibly by acting as a competitive inhibitor [5,8,10]. In contrast to the effect on PC oxidation, however, substitution of the trans double bond in sphingosine with a cis double bond decreased the ability of SM to inhibit the activities of secretory phospholipases A₂ V and X, and had no effect on lecithin:cholesterol acyltransferase (LCAT) activity. Dihydro-SM was more inhibitory than either of the unsaturated analogs for all the phospholipases tested (unpublished data). The cholesterol-binding property of SM is also reduced by the presence of a cis double bond, compared to the natural trans double bond (Fig. 8). Previous studies with short-chain ceramide analogs showed that the apoptotic activity of ceramide was increased by 30% when the natural trans double bond was replaced by a cis double bond [42]. Ceramide containing a cis double bond is not recognized by brain ceramidase [43], indicating that the geometry of the double bond is critical in the cellular metabolism of sphingolipids. Although our studies show a significant enhancement of the antioxidant property of SM by the introduction of a cis double bond in its long chain base, further studies are needed to determine the effect of double bond geometry in membrane sphingolipids of intact cells on other properties such as cell signaling and the structure and function of membrane rafts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by NIH grants HL-68585 (PVS) and HL-083187 (RB). We wish to thank Ms. Buzulagu Aizezi for technical assistance.

Abbreviations

AAPH	2,2'-azo-bis(2-amidinopropane) dihydrochloride
LC/MS	liquid chromatography/mass spectroscopy
LUV	large unilamellar vesicles
MβCD	methyl β-cyclodextrin
PC	phosphatidylcholine
SM	sphingomyelin

References

1. Masserini M, Ravasi D. *Biochim Biophys Acta* 2001;1532:149–161. [PubMed: 11470236]
2. Pike LJ. *J Lipid Res* 2003;44:655–667. [PubMed: 12562849]
3. Jiang XC, Paultre F, Pearson TA, Reed RG, Francis CK, Lin M, Berglund L, Tall AR. *Arterioscler Thromb Vasc Biol* 2000;20:2614–2618. [PubMed: 11116061]

4. Nelson JC, Jiang XC, Tabas I, Tall A, Shea S. *Am J Epidemiol* 2006;163:903–912. [PubMed: 16611667]
5. Subbaiah PV, Liu M. *J Biol Chem* 1993;268:20156–20163. [PubMed: 8376375]
6. Bolin DJ, Jonas A. *J Biol Chem* 1996;271:19152–19158. [PubMed: 8702592]
7. Rye KA, Hime NJ, Barter PJ. *J Biol Chem* 1996;271:4243–4250. [PubMed: 8626769]
8. Gesquiere L, Cho W, Subbaiah PV. *Biochemistry* 2002;41:4911–4920. [PubMed: 11939786]
9. Singh DK, Gesquiere LR, Subbaiah PV. *Arch Biochem Biophys* 2007;459:280–287. [PubMed: 17178097]
10. Singh DK, Subbaiah PV. *J Lipid Res* 2007;48:683–692. [PubMed: 17148553]
11. Koumanov K, Wolf C, Bereziat G. *Biochem J* 1997;326:227–233. [PubMed: 9337873]
12. Subbaiah PV, Sargis RM. *Med Hypotheses* 2001;57:135–138. [PubMed: 11461160]
13. Subbaiah PV, Subramanian VS, Wang K. *J Biol Chem* 1999;274:36409–36414. [PubMed: 10593936]
14. Sargis RM, Subbaiah PV. *Free Radical Biol Med* 2006;40:2092–2102. [PubMed: 16785023]
15. Oborina EM, Yappert MC. *Chem Phys Lipids* 2003;123:223–232. [PubMed: 12691854]
16. Kravaka JM, Li L, Szulc ZM, Bielawski J, Ogretmen B, Hannun YA, Obied LM, Bielawska A. *J Biol Chem* 2007;282:16718–16728. [PubMed: 17283068]
17. Holland WL, Brozinick JT, Wang LP, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts TA, Siesky A, Nelson DH, Karathanasis SK, Fontenot GK, Birnbaum MJ, Summers SA. *Cell Metabolism* 2007;5:167–179. [PubMed: 17339025]
18. Li L, Tang X, Taylor KG, DuPre DB, Yappert MC. *Biophys J* 2002;82:2067–2080. [PubMed: 11916863]
19. Brockman HL, Momsen MM, Brown RE, He L, Chun J, Byun HS, Bittman R. *Biophys J* 2004;87:1722–1731. [PubMed: 15345551]
20. Sargis RM, Subbaiah PV. *Biochemistry* 2003;42:11533–11543. [PubMed: 14516205]
21. Herold P. *Helv Chim Acta* 1988;71:354–362.
22. Chun J, Byun HS, Bittman R. *J Org Chem* 2003;68:348–354. [PubMed: 12530859]
23. McEwen AB, Guttieri MJ, Maier WF, Laine RM, Shvo Y. *J Org Chem* 1983;48:4436–4438.
24. Bittman R, Verbicky CA. *J Lipid Res* 2000;41:2089–2093. [PubMed: 11108743]
25. Marinetti GV. *J Lipid Res* 1962;3:1–20.
26. Chen CH, Albers JJ. *J Lipid Res* 1982;23:680–691. [PubMed: 6811681]
27. Bligh EG, Dyer WJ. *Can J Biochem Physiol* 1959;37:911–917. [PubMed: 13671378]
28. Aringer L, Eneroth P, Nordstrom L. *J Lipid Res* 1976;17:263–272. [PubMed: 932558]
29. Niu SL, Mitchell DC, Litman BJ. *Biochemistry* 2005;44:4458–4465. [PubMed: 15766276]
30. Hahnel D, Huber T, Kurze V, Beyer K, Engelmann B. *Biochem J* 1999;340:377–383. [PubMed: 10333478]
31. Mathiesen L, Malterud KE, Sund RB. *Free Radic Biol Med* 1997;22:307–311. [PubMed: 8958155]
32. Ravandi A, Kuksis A, Shaikh NA. *J Biol Chem* 1999;274:16494–16500. [PubMed: 10347212]
33. Barenholz, Y. Sphingomyelin-lecithin balance in membranes: Composition, structure, and function relationships. In: Shinitzky, M., editor. *Physiology of Membrane Fluidity*. CRC press; Boca Raton: 1984. p. 131-173.
34. Bittman R, Kasireddy CR, Mattjus P, Slotte JP. *Biochemistry* 1994;33:11776–11781. [PubMed: 7918394]
35. Kuikka M, Ramstedt B, Ohvo-Rekila H, Tuuf J, Slotte JP. *Biophys J* 2001;80:2327–2337. [PubMed: 11325733]
36. Thomas, CE. Approaches and rationale for the design of synthetic antioxidants as therapeutic agents. In: Packer, L.; Cadenas, E., editors. *Handbook of Synthetic Antioxidants*. Marcel Dekker; New York: 1997. p. 1-52.
37. Wiseman H, Quinn P. *Free Radic Res* 1994;21:187–194. [PubMed: 7981789]
38. Wiseman H, Quinn P, Halliwell B. *FEBS Lett* 1993;330:53–56. [PubMed: 8370459]
39. Burkitt MJ. *Arch Biochem Biophys* 2001;394:117–135. [PubMed: 11566034]

40. Zommara M, Tachibana N, Mitsui K, Nakatani N, Sakono M, Ikeda I, Imaizumi K. *Free Radical Biol Med* 1995;18:599–602. [PubMed: 9101253]
41. Sindelar PJ, Guan Z, Dallner G, Ernster L. *Free Radical Biol Med* 1999;26:318–324. [PubMed: 9895222]
42. Kishida E, Kasahara M, Takagi Y, Matsumura M, Hayashi T, Kobayashi S, Masuzawa Y. *J Lipid Mediat Cell Signal* 1997;16:127–137. [PubMed: 9246602]
43. El Bawab S, Usta J, Roddy P, Szulc ZM, Bielawska A, Hannun YA. *J Lipid Res* 2002;43:141–148. [PubMed: 11792733]

Fig. 1.

Effect of SM analogs on the oxidation of 16:0-18:2 PC in the presence of Cu^{2+} . PC liposomes (LUVs) containing no SM (PC control), or with 20 mol% of the indicated SM, were prepared by the extrusion method as described in the text. Oxidation of the liposomes (containing 240 nmol of PC and 48 nmol of SM where indicated) was carried out at 37 °C in the presence of 50 μM Cu^{2+} in a spectrophotometer, and the conjugated diene formation was measured by the increase in absorbance at 234 nm. The lag times were: 21 min, 50 min, 140 min, and 25 min respectively for PC control, trans SM, cis SM, and dihydro SM. The slopes ($\Delta A_{234}/\text{min} \times 10^3$) were: 2.050, 1.623, 0.215, and 1.123, respectively for PC control, trans SM, cis SM, and dihydro SM. The results shown are from one typical experiment. Similar data were obtained from at least 3 other experiments.

Fig. 2.

Effect of SM concentration on PC oxidation. Liposomes containing 16:0-18:2 PC and the indicated mol% of cis or trans (egg) SM were prepared by extrusion through the polycarbonate filters as described in the text. The oxidation of the liposomes was carried out using 240 nmol of PC per reaction, and 100 μ M CuCl₂ at 37 °C. The slopes of increase in A₂₃₄ were calculated from the linear portion of the curves, and all values are expressed as % of the slope obtained with PC alone (no SM). The values shown are mean $\pm$ S.D of 3–4 experiments.

Fig. 3.

Effect of Cu^{2+} concentration on the inhibition of PC oxidation by SM analogs. 16:0-18:2 PC liposomes containing either cis- or trans-SM at 20 mol% were oxidized in the presence of increasing amounts of Cu^{2+} in order to determine whether the differences between the two SMs is due to the effective concentration of Cu^{2+} available. The slopes of the reaction ($\Delta A_{234}/\text{min} \times 10^3$), calculated from the maximal linear portion of each graph, were as follows: PC alone (200 μM Cu^{2+}): 2.057; trans-SM (50 μM Cu^{2+}): 1.595; trans-SM (100 μM Cu^{2+}): 1.715; trans-SM (200 μM Cu^{2+}): 1.75; cis-SM (50 μM Cu^{2+}): 0.219; cis-SM (100 μM Cu^{2+}): 0.298; cis-SM (200 μM Cu^{2+}): 0.646.

Fig. 4.

Effect of SM unsaturation on AAPH-mediated oxidation of PC. 16:0-18:2 PC liposomes (LUVs) containing 20 mol% of either egg (trans) SM, cis-SM, or dihydro-SM were prepared by the extrusion method as described in the text. The liposomes were incubated with 0.1 mM AAPH at 37 °C, and the conjugated diene formation was monitored by the absorbance at 234 nm. The slopes of the reaction were calculated from the regression lines drawn in the maximal linear range of each curve. The lag periods were: PC alone: 23 min; with egg SM: 32 min; with cis-SM: 93 min; and with dihydro-SM: 34 min.

Fig. 5. Effect of cis unsaturation in the *N*-acyl group of SM on Cu^{2+} -mediated oxidation of PC. Liposomes containing PC alone, or with the addition of 20 mol% of the indicated SM were prepared by membrane extrusion. The incubation mixture (at 37 °C) contained 240 nmol of PC, 48 nmol of the indicated SM, and 50 μM Cu^{2+} , and the formation of conjugated dienes was monitored at by the absorbance at 234 nm. The slopes, calculated from the maximal linear portion of each graph ($\Delta A_{234}/\text{min} \times 10^3$), were: PC control: 0.955; with egg SM: 0.751; with *N*-(C18:1 $\Delta 9$ cis)-SM: 0.070; with *N*-(C18:0)-SM: 0.704; and with *N*-(C16:1 $\Delta 4$ cis)-SM: 0.877.

Fig. 6.

Effect of SM analogs on the oxidation of cholesterol by FeSO_4 /ascorbate. [^{14}C]-Labeled cholesterol liposomes containing the different SM analogs were oxidized in the presence of 0.5 mM FeSO_4 /5mM ascorbate for the indicated periods of time, and the labeled cholesterol oxidation products were measured as described in the text.

Fig. 7.

Effect of SM analogs on the oxidation of cholesterol by AAPH. 18:1-18:1 PC liposomes containing 10 mol% 4- ^{14}C cholesterol, and 20 mol% of the indicated SM were prepared by cholate dialysis as described in the text. The liposomes were oxidized in the presence of 10 mM AAPH at 37 °C for the indicated periods of time, and the percent of label in the total oxidation products was determined after TLC separation of the products, as described in the text. The values shown are mean $\pm$ S.D. of 3 experiments.

Fig. 8.

Effect of double bond geometry in SM on binding of SM to cholesterol. Liposomes containing 16:0-18:2 PC and the indicated SM analog at a PC:SM molar ratio of 4:1 were incubated with [^{14}C]-cholesterol-M β CD complex for 2 h at 37 °C, and the percent of labeled cholesterol transferred to the liposomes was determined as described in the text. The values are expressed as the % increase in transfer relative to the control liposomes which contained only PC. The values shown are mean $\pm$ S.D. of 4 experiments. * $p < 0.05$, compared to egg SM (*trans* N-C16 SM).

Fig. 9.

Fates of PC and SM during Cu^{2+} -mediated oxidation

Liposomes containing 16:0-18:2 PC in the presence of cis or trans SM at PC: SM molar ratio of 4:1 were oxidized in presence of 50 μM CuCl_2 at 37 °C. Aliquots of the reaction mixture were taken out at the indicated times, extracted by Bligh and Dyer procedure after adding 17:0-17:0 PC as internal standard, and analyzed by LC/MS as described in the text.

Chart 1.