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Protection of membrane cholesterol by sphingomyelin against free radical-mediated oxidation

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Abstract

Although the free radical-mediated oxidation of free cholesterol (FC) is critical in the generation of regulatory sterols and in atherogenesis, the physiological regulation of this process is poorly understood. We tested the hypothesis that sphingomyelin (SM), a major phospholipid of cell membranes, which is closely associated with FC, protects FC against oxidation, because of its unique structure, and affinity to the sterol. We employed phosphatidylcholine (PC) liposomes containing varying amounts of SM, and either radioactive FC or a fluorescent analog, dehydroergosterol (DHE), and determined the oxidative decay of the sterol in presence of 2,2' azo bis(2-amidinopropane hydrochloride) (AAPH). Incorporation of 25 mol% of SM in the liposomes inhibited the oxidation of FC or DHE by up to 50%. This inhibition was specific for SM among phospholipids, and was abolished by sphingomyelinase treatment. SM was not degraded during the oxidation reaction, and its effect was not dependent upon the nature of the oxidizing agent, because it also inhibited sterol oxidation by FeSO₄/ascorbate, and by cholesterol oxidase. These studies show that SM plays a physiological role in the regulation of cholesterol oxidation by free radicals.

Keywords

cholesterol oxidation; oxysterol; sphingomyelinase; dehydroergosterol; AAPH; cholesterol oxidase; FeSO₄/ascorbate

Abbreviations used

AAPH, 2,2'-azo-bis(2-amidinopropane) dihydrochloride; DHE, dehydroergosterol; FC, free (unesterified) cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; SMase, sphingomyelinase

Introduction

Sphingomyelin (SM) is a major phospholipid in cell membranes and lipoproteins of all animals. Its concentration varies considerably among different cells, and even among membranes of the same cell [1,2]. An intriguing feature of SM distribution is that it parallels the distribution of free (unesterified) cholesterol (FC). For example, both these lipids are concentrated in the plasma membrane of the mammalian cells [3], and are critical components of the membrane rafts which are implicated in a wide variety of cellular functions [4]. Among the lipoproteins,

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both FC and SM are more concentrated in low density lipoproteins (LDL), when compared to high density lipoproteins [5,6]. Even among the LDL subfractions, the distributions of SM and FC are similar to each other, with the more buoyant subfractions containing higher concentrations of SM and FC per unit phospholipid [5,7,8]. Whereas the two lipids are known to physically interact with each other through hydrogen bonding and van der Waals forces [1,9], the physiological significance of their co-localization in biological systems is not clear. We have previously reported that SM is an inhibitor of peroxidation of polyunsaturated fatty acids in the phospholipids, possibly because of its ability to inhibit the propagation of peroxy radicals [10]. Compared to the interaction of SM with other phospholipids in the membrane, its interaction with cholesterol is stronger [1,2,11]. This preferential interaction is also supported by the existence of the detergent-resistant membrane domains (rafts) which are rich in SM and FC, but relatively poor in glycerophospholipids [4]. It is also well established that SM restricts the movement of FC between cellular membranes and lipoproteins, and decreases its availability to external acceptors [12]. Because of their close association, it is possible that SM could also affect the oxidation of FC in the membranes and lipoproteins. In this study we investigated the effect of SM on the oxidizability of FC by free radicals, employing defined liposomes containing radioactive or fluorescent sterols.

Cholesterol is oxidized by both enzymatic and non-enzymatic pathways in animal systems. The enzymatic pathways are especially important in bile acid synthesis, which is the principal disposal mechanism for excess cholesterol from the body. However the non-enzymatic and free radical-mediated pathways of cholesterol oxidation may be more relevant to the pathological consequences of cholesterol oxidation. In general, cholesterol is less oxidizable by the free radicals compared to the polyunsaturated fatty acids, because of its planar, saturated structure. Once generated, however, the products of cholesterol oxidation have more potent and diverse biological effects. In fact they have been implicated in the etiology of several diseases, including atherosclerosis, cancer, and neurological disorders [13,14]. Individual oxysterols have been shown to affect apoptosis [15], gene regulation [16], chemotaxis [17], and lipid homeostasis in cells and the body [14,16,18]. Hence it is important to understand the physiological factors that regulate the generation of oxysterols in the body. The studies reported here show that sphingomyelin, the companion lipid of cholesterol, is a physiological inhibitor of oxysterol generation.

Materials and Methods

Materials

[4-¹⁴C] cholesterol (54 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc (St. Louis, MO). AAPH (2,2'-azo-bis(2-amidinopropane) dihydrochloride) was obtained from Wako Pure Chemical Industries (Richmond, VA). Egg phosphatidylcholine (PC), 18:1-18:1 PC, egg phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), and egg SM were purchased from Avanti Polar Lipids (Alabaster, AL). Dehydroergosterol (DHE), unlabeled cholesterol, cholesterol oxidase (*Streptomyces sp*), and sphingomyelinase (SMase) C (*Staphylococcus aureus*) (EC 3.1.4.12) were purchased from Sigma Chemical Co (St. Louis, MO). Histidine-tagged recombinant SMase D (*Clostridium pseudotuberculosis*) (EC 3.1.4.41) was purified by affinity chromatography from transfected *E. Coli*, as described previously [19].

Liposome preparations

Liposomes containing 18:1-18:1 PC (or egg PC) and either radiolabeled FC, or unlabeled FC and DHE, were prepared by the cholate dialysis procedure [20] in the absence of apoprotein AI. Briefly, chloroform solutions of PC (2 μ mol) and either FC (0.2 μ mol) or FC+DHE (0.1 μ mol each) 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 lipids, 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 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 dialyzed sample was adjusted to 1.0 ml volume 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. Where indicated, egg SM was included either as a supplement or as a partial substitute for PC. In one experiment, egg PE or brain PS substituted for 25 mol% of PC.

SMase treatments

Liposomes (200 μ l) were incubated with 0.2 units of SMase C or 0.5 μ g of recombinant SMase D in the presence of 0.8 mM each of $MgCl_2$ and $MnCl_2$ for 2 h at 37 °C. The enzyme reaction was stopped by the addition of 2.5 mM EDTA. Control samples were treated identically except for the absence of the enzyme. The extent of SM hydrolysis was determined in separate samples by estimation of lipid phosphorus, after TLC separation of the lipid extract [19]. Typically, about 70–80% of SM was hydrolyzed under these conditions by both the enzymes.

Oxidation of [^{14}C] cholesterol

Liposomes (100 μ l) containing [4- ^{14}C] cholesterol were incubated either with 10 mM AAPH, or with 0.5 mM $FeSO_4$ /5 mM ascorbate, for varying periods of time at 37 °C, in a final volume of 0.4 ml. The reaction was stopped by extraction of the lipids by Bligh and Dyer procedure [21]. The total lipid extract was applied to a silica gel TLC plate, and 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 FC spot was identified with the help of a standard run on the same plate. The radioactivity below the FC spot 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 -OH and 7- β -OH cholesterol, based on the R_f values in literature [22]. The less polar product (migrating between FC and the origin) was similarly identified as predominantly 7-oxocholesterol. No radioactivity was found above the FC 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-oxo cholesterol spot, and the rest in the origin (7- α -OH and 7- β -OH cholesterol). The oxidation of cholesterol was calculated as the percent of initial FC counts found in all of the oxidation products (2 bands from TLC plate and the aqueous layer).

Oxidation of DHE

The oxidative decay of DHE fluorescence was measured in an ISS PC-1 spectrofluorometer equipped with a thermostated 3-cell holder and a magnetic stirrer. Liposomes (200 μ l, containing 0.4 μ mol PC) were diluted with Tris-NaCl buffer to 3 ml in the cuvette, and allowed to equilibrate at 37 °C for 15 min, while stirring. AAPH (2–5 mM) was then added in a small volume of Tris-NaCl buffer (40 μ l), and the incubation continued at 37 °C for up to 90 min, with continuous stirring of the sample. Fluorescence intensity was recorded at 8 sec intervals with an excitation wavelength of 324 nm (band-pass of 0.5 nm) and an emission wavelength of 376 nm (band-pass of 2 nm). The fluorescence values were normalized by expressing them as percent of the initial fluorescence (0 min). The 0 min value was usually set at 20 sec after AAPH addition, because of an immediate drop in fluorescence that occurs possibly due to quenching by AAPH. The data were fit to an exponential equation using the Math function of

SlideWrite (Advanced Graphics Software). The time required for the loss of 25% of initial fluorescence was calculated from the derived equation. A 25% reduction was chosen to facilitate statistical comparisons among different experiments because the extrapolated $t_{1/2}$ values (time required for 50% decrease in fluorescence) were infinitely long in most of the samples containing SM.

Phospholipid determination

To determine the degradation of PC and SM during oxidation, the lipid extract from the reaction mixture was separated on silica gel TLC plate with the solvent system of chloroform/methanol/water (65: 25: 4 v/v), and the spots were visualized by exposure to iodine vapors. The PC and SM spots were identified with the help of standards run on the same plate, and the lipid phosphorus was determined by the modified Bartlett procedure [23]. The oxidized PC was present mainly between the origin and the SM spot. A small amount of oxidized PC also migrated with SM. The phosphorus values in SM were therefore corrected for this by subtracting the value of the control samples which were oxidized in the absence of SM.

Statistics

The significance of difference between experimental values was determined by Student's *t* test (2 tailed, paired *t*), using Microsoft Excel. Values were considered significantly different at $p < 0.05$.

Results

1. Oxidation of labeled cholesterol

The effect of incorporation of SM into liposomes on the oxidation of labeled cholesterol is shown in Fig.1. Liposomes containing 18:1-18:1 PC and [^{14}C] cholesterol (10 mol% of PC) were prepared in the absence or presence of 25 mol% of SM. The liposomes were oxidized with 10 mM AAPH at 37 °C for the indicated period of time. The total lipids were extracted and the radioactivity in the un-oxidized cholesterol and oxidized products was determined after TLC separation, as described in Methods. The oxidized products separated into two bands on the plate (a polar band at the origin, and a less polar band above the origin). The radioactivity in the aqueous layer and the two bands of oxidized cholesterol were combined to calculate the total percent of cholesterol oxidized. As shown in Fig.1, the presence of 25 mol% SM in the liposome resulted in about a 50% inhibition of cholesterol oxidation, measured either as the percent of cholesterol remaining intact, or as the percent formation of oxidation products. The inhibition was statistically significant at all the time points studied ($p < 0.05$).

Although we have not conclusively identified the oxidation products in this study, the major products formed by AAPH oxidation of cholesterol are known to be 7-oxo (keto) cholesterol, 7 α - and 7 β - hydroxy cholesterol, and 5,6-epoxy cholesterol [24,25]. Based on the R_f values reported in the literature [22], we tentatively identified the polar (origin) spot as a mixture of 7 α - and 7 β - hydroxy cholesterol, and the less polar spot as predominantly 7-oxo cholesterol. After 4h oxidation with 10 mM AAPH, approximately 24% of the total radioactivity was present in the 7-oxo cholesterol spot and about 3% of the total in the 7-hydroxy cholesterol spot. Less than 0.2% of the total radioactivity was found in the aqueous layer. This distribution of the radiolabel is in agreement with the literature values for AAPH-oxidized cholesterol [25]. The presence of SM inhibited the formation of all products to the same extent (results not shown).

2. Specificity of SM effect

To determine whether the inhibition by SM is specific for this phospholipid, we compared the effect of incorporation of egg PE and brain PS with that of SM. When incorporated at 25 mol % of total phospholipid, only SM inhibited cholesterol oxidation (Fig. 2). The inhibition was much greater when labeled cholesterol was incorporated into pure SM liposomes, although these liposomes were considerably more turbid compared to the PC-containing liposomes. Although previous studies [25] reported some inhibition of cholesterol oxidation by egg PE, both PE and PS modestly stimulated the oxidation in our experimental system. The reason for this discrepancy is not clear, but there are several differences in the experimental conditions between the two studies. For example, the cholesterol : phospholipid ratio (1:10) is much lower in our system compared to the previous study (~ 1:2). Also the PC matrix used here (18:1-18:1 PC) is less unsaturated than in the previous study, which utilized soybean PC.

3. Effect of oxidizing agent

To determine whether the protective effect of SM is specific for the oxidation reagent used, we also oxidized the labeled cholesterol liposomes in presence of FeSO_4 /ascorbate. Liposomes containing egg PC $\pm$ 25 mol% SM were oxidized for 0–16 h at 37 °C in presence of 0.5 mM FeSO_4 /5 mM ascorbate, and the radioactivity in cholesterol and its oxidation products was determined as described above. Although there was a lag in the oxidation of cholesterol by this procedure, the protection of oxidation by the presence of 25 mol% SM was clearly evident at 16 h (Fig. 3). These results show that SM inhibits cholesterol oxidation irrespective of the source of free radicals.

4. Fate of SM during oxidation

To determine whether SM is degraded during the reaction with oxidizing agents, we studied the effect of oxidation on SM levels by determining the lipid phosphorus in SM after TLC separation from the lipid mixture. In this experiment the liposomes were prepared with egg PC in order to simultaneously follow the oxidation of PC. After oxidation with 10 mM AAPH for 4 h, the total lipids were extracted, and half of the sample was used for determination of cholesterol oxidation as described above, and the other half was used for the separation of phospholipids on TLC plate, as described in Methods. Spots corresponding to SM, oxidized PC, and PC were scraped and their lipid phosphorus was determined by the modified Bartlett procedure [23]. Since a minor amount of oxidized PC migrated with SM in this solvent system, we corrected the SM values by subtracting the control values (sample oxidized in the absence of SM). As shown in Fig. 4, the oxidation of labeled cholesterol was inhibited significantly (21.6% oxidation compared to 45.3% oxidation in control, in 4 h) in the presence of 25 mol% of SM. However the quantity of SM was not altered significantly. The amount of PC decreased by about 25% during the oxidation in the absence of SM, and by 18% in the presence of 25 mol% SM. These results show that while SM inhibited the oxidation of both FC and PC, it was not degraded during the oxidation.

5. Effect of SM on the oxidation of a fluorescent analog of cholesterol

In order to monitor the oxidation of cholesterol continuously under milder conditions than used above, we studied the fluorescence decay of DHE in the presence of AAPH in all subsequent experiments. DHE is a naturally occurring sterol that has physicochemical characteristics similar to cholesterol, and is completely miscible with cholesterol in lipid structures [26]. It has been shown to faithfully trace the transport and metabolism of cellular cholesterol [27]. Therefore, the fluorescence decay of DHE under oxidative conditions should reflect the oxidation of cholesterol, although it is relatively more sensitive to oxidizing agents than cholesterol. Therefore, 18:1-18:1 PC liposomes were prepared as described above for the radioactive cholesterol studies, but using unlabeled cholesterol, and replacing 50% of

cholesterol with DHE (PC/FC/DHE at molar ratio of 100:5:5). As shown in Fig. 5, incorporation of SM into the liposomes dose-dependently inhibited the oxidative decay of DHE fluorescence in the presence of 2 mM AAPH. The loss of fluorescence due to photobleaching (in the absence of AAPH) was less than 5% under the experimental conditions (top curve). While this work was in progress, Olsher et al [28] published a study on the use of DHE to study the free radical-induced oxidation of sterols and the effect of sterol superlattice structure, supporting the validity of this assay. It should, however, be pointed out that the oxidation of cholesterol itself was negligible under the conditions used here (2 mM AAPH, <1 h oxidation at 37 °C).

6. SM substitution versus SM supplementation

In the studies described above (Fig.5), the PC and sterol concentrations in the liposomes were kept constant, while incorporating additional SM where indicated, resulting in an increased phospholipid/sterol ratio. It is, therefore, possible that the inhibition of oxidation observed in the presence of SM was either due to dilution of the sterol, or an inhibition of PC oxidation [10] which in turn could affect sterol oxidation. To test this possibility, we prepared liposomes in which the total phospholipid was kept constant by substituting 50% of PC with SM (SM-substituted liposomes) and compared them with liposomes in which PC concentration was held constant and SM was added at 50 mol% of PC (SM-supplemented liposomes), thereby increasing the total phospholipid/sterol ratio. The concentration of sterol (FC and DHE) was the same in both liposomes. As shown in Fig. 6, the fluorescence decay curves of the SM-substituted and SM-supplemented liposomes were similar to each other, but differed significantly from the SM-free liposomes. These results show that the presence of SM, rather than a change in the total phospholipid/sterol ratio, was responsible for the observed inhibition of sterol oxidation. It may be pointed out that since 18:1-18:1 PC was used in these studies, the extent of PC oxidation is minimal under the conditions employed, and therefore it is unlikely that the observed effect of SM was due to the secondary effects on PC oxidation. Furthermore, similar results were obtained when egg PC was used in place of 18:1-18:1 PC (results not shown). These results are in general agreement with the results of Maeba et al [25], who reported that the unsaturation of the phospholipids has no significant effect on the extent of cholesterol oxidation by AAPH.

7. Quantification of sterol oxidation rates

The fluorescence decay curves of DHE showed exponential rates of decay with the r^2 values >0.95. Since the predicted $t_{1/2}$ values (time required for 50% loss of fluorescence) were infinitely long in the presence of SM in most experiments, $t_{3/4}$ values (time required for 25% loss of fluorescence) were calculated from the curve-fit equations. As shown in Fig. 7, this parameter is significantly increased by the presence of either 25 mol% or 50 mol% SM ($n=8$, paired t test).

8. Structural requirement for SM inhibition of oxidation

We next studied whether the inhibition of sterol oxidation required the intact structure of SM, by treating the SM-containing liposomes (as well as the SM-free control liposomes) with either SMase C (Fig. 8) or SMase D (Fig. 9) before oxidation with AAPH. SMase C hydrolyzes SM to ceramide and phosphorylcholine, whereas SMase D hydrolyzes it to ceramide phosphate and free choline. Treatment of SM-liposomes with SMase C prior to initiation of oxidation not only prevented the effect of SM, but also activated the oxidation of DHE, when compared to the SM-free liposomes (Fig. 8). Treatment with SMase D, however, only abolished the SM effect, without further activation of the oxidation (Fig. 9). The difference between the effects of the two enzymes is most likely due to the effects of ceramide generation (in the case of SMase C), which is known to disrupt the bilayer structure [29], and to displace cholesterol

from lipid rafts [30]. Ceramide phosphate that is generated by SMase D, in contrast, is a phospholipid that likely maintains the bilayer structure. Nevertheless, these results show that an intact structure of SM is necessary for its inhibition of sterol oxidation, similar to the inhibition of PC oxidation [10]. It should be mentioned that the absolute rates of oxidation in the two experiments differ because the studies were performed with different batches of liposomes. Despite this difference, the effect of SM, as well as the reversal by SMases were highly reproducible.

9. Effect of SM on enzymatic oxidation of DHE

In addition to the free radical-mediated oxidation of DHE, we studied the effect of SM on the oxidation of DHE by cholesterol oxidase. Although it does not occur in mammalian systems, this enzyme has been used extensively as a probe for membrane cholesterol [31–33]. As shown in Fig. 10, the oxidation of DHE by cholesterol oxidase was also significantly inhibited by the presence of 50 mol% SM. This further supports the validity of DHE as a surrogate for cholesterol, because the effects of SM on its enzymatic oxidation are similar to those reported previously for enzymatic oxidation of cholesterol in cells and lipid monolayers [31] [34].

Discussion

The pathophysiologic significance of oxysterols in mammalian systems is well established [13,14]. Many oxysterols regulate gene expression in cells by acting as ligands for nuclear receptors and sterol responsive element binding proteins [16,35], while others are cytotoxic [36], chemotactic [17] or apoptotic [15]. They have been implicated in the development of atherosclerosis, cancer and neurological disorders [13,14]. Significant amounts of oxysterols are also present in atherosclerotic lesions [13,14]. Although the exact mechanisms of their formation *in vivo* are not fully understood, it is likely that the free radical-mediated oxidation plays a major role, and therefore, the regulation of their production by this pathway is of great importance.

The results presented here provide evidence that free radical-mediated oxidation of cholesterol is regulated by SM, its companion lipid in cell membranes and lipoproteins. These two lipids are known to be distributed co-variantly in cell membranes and lipoproteins, and a strong physical interaction between these two lipids may be one reason for this association [1,2]. The physiological significance of this association, however, is not known, although previous studies showed that depletion of membrane SM by SMase C treatment induces cholesterol to move from the plasma membrane to intracellular membrane or to an exogenous acceptor [2,37]. Interestingly, SMase C treatment also stimulates the oxidation of membrane cholesterol by bacterial cholesterol oxidase [31]. This finding may not, however, be physiologically relevant because cholesterol oxidase is not present in mammalian systems. Moreover, the inhibition of this enzyme activity could be due to a specific effect of SM on the enzyme itself, rather than a general protection of cholesterol against oxidative agents. The protection of cholesterol against free radical-mediated oxidation may, however, be more physiologically relevant since the cell membranes and lipoproteins are continuously exposed to free radicals generated by endogenous systems such as P-450 enzymes and phagocytic cells [38], as well as by exogenous factors such as radiation, diet, drugs, and pollutants [39]. It is important to point out that the bulk of the cellular SM is present in the plasma membrane, and specifically concentrated in the outer monolayer [1–4]. Therefore SM may be one of the first lines of defense against free radical-mediated damage. In other studies from our laboratory, we have shown that SM also protects the membranes against attack by secretory phospholipases [40]. Thus, its location in the outermost monolayer of the cell may be physiologically important in protecting the cells against various types of threat from the external milieu [41]. SM may have similar protective function in the lipoproteins where the oxidation of phospholipids [42] and cholesterol [14] is

known to be an important contributor to the atherosclerotic disease. Although there is some evidence that abnormally high concentration of SM in plasma may increase the risk of atherosclerosis because of increased LDL aggregation [43], the function of SM under normal physiological conditions may actually be anti-atherogenic.

The exact mechanism by which SM inhibits cholesterol oxidation is not yet clear, although several possible mechanisms could be considered. The following points should be taken into account in understanding the mechanism of SM action. i) the intact molecule of SM is necessary for inhibition of cholesterol oxidation, because removal of either choline or choline phosphate by SMase treatment abolished this function. ii) SM does not have the ability to scavenge free radicals or metal ions like other known anti-oxidants. iii) SM has mostly saturated hydrocarbon groups which are resistant to oxidation. There is a *trans* double bond in the sphingosine moiety, but our previous studies showed that the *trans* double bond is 6 times more resistant than the *cis* double bond to free radical-mediated oxidation [44]. The resistance of SM to oxidation is confirmed by the observation that SM is not degraded during the oxidation of FC and PC (Fig. 3). These results therefore show that SM does not have a 'sparing' effect on cholesterol, and that it does not terminate the free radical reaction. iv) The effect of SM is not dependent upon the nature of the oxidizing agent, because it inhibited the oxidation by AAPH, FeSO₄/ascorbate, or cholesterol oxidase. v) Other phospholipids such as PE and PS did not inhibit cholesterol oxidation under the same conditions. Based on these results, and the fact that SM interacts preferentially with FC through the hydrogen bonding and van der Waals forces [1], we propose that SM reduces the oxidizability of FC through its physical effects on the sterol, rather than by affecting the free radical generation or propagation. This is also consistent with the observation that the degradation of SM to ceramide actually stimulated sterol oxidation (Fig. 8), because ceramide is known to displace cholesterol from the membrane rafts [30], and therefore might expose it to free radicals generated by AAPH in the aqueous environment. It is also possible that the saturated acyl groups of SM play an important role, because the incorporation of 16:0-16:0 PC also inhibited the oxidation in the absence of SM (results not shown).

Exactly how the oxidizability of FC is decreased by SM cannot be ascertained from our studies. Recently Olsher et al [28] suggested that the lateral organization of cholesterol in a superlattice structure determines its oxidizability by free radicals. They reported that the rate of cholesterol oxidation exhibits several minima and maxima, which correspond to the critical sterol concentrations predicted for the superlattice formation. These studies, however, were performed at the sterol concentrations ranging from 20 mol% to 45 mol% of phospholipid. In the present study, the sterol concentration was between 6 mol% and 10 mol% of total phospholipid, which is below the critical concentration required for the superlattice formation [45]. It is, therefore, unlikely that variations in the superlattice structure account for the inhibitory effect of SM in our studies. Furthermore, the results in Fig. 6 show that the inhibitory effect of SM was the same whether the sterol concentration was 6.7 mol% (SM-supplemented liposomes) or 10 mol% (SM-substituted liposomes) of the total phospholipid. On the other hand, increasing the SM concentration augmented the inhibition of sterol oxidation (Fig. 5 and Fig. 7). According to the superlattice model, the sterol concentration should be more critical than the composition of the bilayer phospholipids, for sterol oxidizability.

In contrast to the superlattice model which predicts that increasing the number of bilayer components decreases the packing density [28], other studies have shown that the presence of SM actually increases the packing density of the membranes [46]. Increasing the packing density is known to inhibit the enzymatic oxidation of cholesterol by cholesterol oxidase [46], although it is not known whether the free radical-mediated oxidation is also sensitive to surface pressure. Studies by Suwa et al [47] showed that membrane fluidity plays an important role in the oxidation of cholesterol, and since SM decreases the fluidity of the membrane [1, 2], this could be another possible mechanism by which it inhibits cholesterol oxidation.

Osborne and Yappert [48], on the other hand, have proposed that the hydrogen bonding between neighboring SM molecules forms a strong interfacial barrier for the penetration of the oxidizing agents, and that this barrier effect may be more important than the fluidity effect of SM, in protecting PC from oxidation. Since the hydrogen bonding between SM and FC molecules is known to be strong [1], this could be an additional contributor to the inhibitory effect of SM on cholesterol oxidation. Whatever the mechanism, our results show that SM is an important physiological inhibitor of free radical-mediated cholesterol oxidation, and hence may protect the cells and lipoproteins from the deleterious effects of oxysterols.

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Figure 1. Effect of SM on the oxidation of [^{14}C] cholesterol by AAPH

A: Effect on the formation of oxidation products. B: Effect on the disappearance of labeled cholesterol

Liposomes containing 18:1-18:1 PC and [^{14}C] cholesterol at a molar ratio of 10:1 were incubated with 10 mM AAPH at 37 °C for various periods of time. Where indicated, egg SM was incorporated into the liposomes at 25 mol% of PC, keeping the PC and cholesterol concentrations constant. The oxidation was stopped by lipid extraction, the total lipid extract was separated on silica gel TLC plates, and the radioactivity in the un-oxidized and oxidized cholesterol spots was determined as described in the text. * $p < 0.05$, ** $p < 0.005$, compared to control (paired t test, $n=3$).

Figure 2. Specificity of SM effect

[4-¹⁴C]-FC was incorporated at 10 mol% of total phospholipid into liposomes containing either 100% di-18:1 PC, 100% SM, or a mixture of phospholipids as indicated. The oxidation was carried out in the presence of 10 mM AAPH for 4 h at 37 °C. The radioactivity in the oxidation products was determined as described in the text. * $p < 0.01$; ** $p < 0.001$, compared to 100% PC (Student's t test, $n=3$).

Figure 3. Effect of SM on the oxidation of [^{14}C] cholesterol by FeSO_4 /ascorbate

Liposomes containing egg PC and [^{14}C] cholesterol at a molar ratio of 10:1 or egg PC, egg SM, and [^{14}C] cholesterol at molar ratio of 7.5:2.5:1, were treated with 0.5 mM FeSO_4 and 5 mM ascorbate for the indicated periods of time at 37 °C. The total lipids were extracted and the radioactivity in cholesterol and the oxidation products was determined after TLC separation, as described in Methods. The values shown are averages of duplicate determinations from a single experiment.

Figure 4. Effect of AAPH oxidation on SM

Top: Effect of AAPH on oxidation of labeled cholesterol. **Bottom:** Effect of AAPH on phospholipid composition. Liposomes containing egg PC and [4-¹⁴C] cholesterol at a molar ratio of 10:1 or egg PC, egg SM, and [4-¹⁴C] cholesterol at molar ratio of 7.5:2.5:1 were oxidized in presence of 10 mM AAPH for 4h at 37 °C. After lipid extraction, one half of the extract was used for the determination of cholesterol oxidation products as described in Figures 1–3, and the other half was used for phospholipid determination. The phospholipids were separated on silica gel TLC plates with the solvent system of chloroform: methanol: water (65: 25: 4 v/v) and the spots corresponding to oxidized PC, SM, and PC were scraped and their lipid phosphorus determined as described in the text. The values shown mean ± SD of triplicate

samples from one experiment. Note that the initial concentration of PC is lower (by 25%) in the SM-containing liposomes, compared to the Control liposomes.

Figure 5. Effect of SM on the oxidation of DHE in PC liposomes

Liposomes containing 18:1-18:1 PC, FC and DHE were prepared with the molar ratio of 100:5:5, and were oxidized in the presence of 2 mM AAPH at 37 °C in the cuvette of a spectrofluorometer. The fluorescence intensity was measured at 8 sec intervals using an excitation wavelength of 324 nm and emission wavelength of 376 nm. Where indicated, egg SM was included in the liposomes at 25 mol% or 50 mol% of PC. The PC, FC, and DHE concentrations were held constant in all samples.

Figure 6. Effect of SM substitution versus SM supplementation

The SM-free liposomes (A) contained 18:1-18:1 PC, FC, and DHE at a molar ratio of 100:5:5. In B, egg SM replaced 50 mol% of the PC (SM substituted), while in C, 50 mol% of egg SM was added without reducing PC concentration (SM supplementation). All liposomes were oxidized in the presence of 5 mM AAPH at 37 °C, and the fluorescence decay was recorded as described in the text.

Figure 7. Effect of SM on the rate of DHE oxidation in liposomes

Liposomes in which 0%, 25 mol%, or 50 mol% of PC (18:1-18:1 PC) was replaced by egg SM were prepared by the cholate dialysis procedure, as described in the text, and were oxidized in the presence of 5 mM AAPH at 37 °C. Data from each fluorescence decay curve (as seen in Fig.5 and Fig. 6) were fit to an exponential equation using SlideWrite (Advanced Graphics Software), and the time required for 25% loss of initial fluorescence was calculated from the derived equation. Values shown are means $\pm$ SEM of 8 experiments. Statistical significance between control (No SM) and experimental values was determined by Student's *t* test (paired *t*).

Figure 8. Reversibility of the effects of SM by SMase C

SM-containing liposomes (200 μ l) were treated with SMase C from *S. aureus* (0.2 units) in the presence of 0.8 mM MnCl_2 and 0.8 mM MgCl_2 for 2 h, and the enzyme reaction was stopped by the addition of 2.5 mM EDTA. SM-free and SM-containing liposomes were also pre-incubated with the metal ions and EDTA in the absence of SMase C. All samples were then oxidized in the presence of 5 mM AAPH, and the fluorescence decay of DHE was measured as described in the text.

Figure 9. Reversibility of SM effect by SMase D

SM-containing liposomes (200 μ l) were treated with recombinant SMase D (0.5 μ g) in the presence of 0.8 mM each of MnCl_2 and MgCl_2 for 2 h, and the enzyme reaction was stopped by the addition of 2.5 mM EDTA. They were then oxidized by 5 mM AAPH and the fluorescence decay of DHE was recorded as described in the text. SM-free and SM-containing liposome control were also treated identically, but without SMase D.

Figure 10. SM inhibition of DHE oxidation by cholesterol oxidase

Liposomes containing egg PC: FC: DHE at the molar ratio of 100:5:5 were incubated with 5 units of cholesterol oxidase at 37 °C in the fluorometer cuvette, and the fluorescence intensity recorded at 8 sec intervals (excitation 324 nm, emission 376 nm).