Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy

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The mechanism of action of 2-hydroxyoleic acid (2OHOA), a potent antitumor compound, has not yet been fully elucidated. Here, we show that human cancer cells have markedly lower levels of sphingomyelin (SM) than nontumor (MRC-5) cells. In this context, 2OHOA treatment strongly augments SM mass (4.6-fold), restoring the levels found in MRC-5 cells, while a loss of phosphatidylethanolamine and phosphatidylcholine is observed (57 and 30%, respectively). The increased SM mass was due to a rapid and highly specific activation of SMS synthases (SMS). This effect appeared to be specific against cancer cells as it did not affect nontumor MRC-5 cells. Therefore, low SM levels are associated with the tumorigenic transformation that produces cancer cells. SM accumulation occurred at the plasma membrane and caused an increase in membrane order and lipid raft packing in model membranes. These modifications would account for the observed alterations by 2OHOA in the localization of proteins involved in cell apoptosis (Fas receptor) or differentiation (Ras). Importantly, SMS inhibition by D609 diminished 2OHOA effect on cell cycle. Therefore, we propose that the regulation of SMS activity in tumor cells is a critical upstream event in 2OHOA antitumor mechanism, which also explains its specificity for cancer cells, its potency, and the lack of undesired side effects. Finally, the specific activation of SMS explains the ability of this compound to trigger cell cycle arrest, cell differentiation, and autophagy or apoptosis in cancer cells.

 anticancer | membrane-lipid therapy | lung cancer | membrane lipids

The potent antitumor compound 2-hydroxyoleic acid (2OHOA) (Minerval®) acts against cancer by inducing cell cycle arrest (1–3), followed by apoptosis in human leukemia cells (4) or differentiation and autophagy in the case of human glioma cells. Despite the potency of 2OHOA against cancer, it is a safe nontoxic compound with IC50 values in nontumor cells 30- to 150-fold greater than in tumor cells (4). The high efficacy and low toxicity of this fatty acid produce a wide therapeutic window that can only be the consequence of a highly specific mechanism of action, the molecular bases of which have, in part, been elucidated here.

The 2OHOA compound was designed rationally to reproduce the antitumor effect of anthracyclines via interactions with the plasma membrane and the ensuing modifications in cell signaling (5), without unspecific interactions with other cell targets. It is known that 2OHOA binds to membranes and modifies the biophysical properties of the bilayer, the first target encountered by this synthetic lipid (6). Nevertheless, the regulatory effects of 2OHOA on the composition of cancer cell membranes have yet to be described. In fact, 2OHOA induces changes in the localization and activity of membrane proteins involved in cancer cell proliferation, differentiation, and survival, such as the Fas receptor (4), PKC (3), as well as cyclins, cyclin-dependent kinases (CDKs), caspases, E2F-1 and dihydrofolate reductase (DHFR)(1, 2). Interestingly, a similar mechanism is described for edelfosine, a synthetic ether lipid with a high apoptotic activity that also induces reorganization of membrane rafts, FasR capping, and cell apoptosis (7).

Sphingomyelin (SM) is a key component of the plasma membrane that interacts with cholesterol and glycerophospholipids, thereby participating in the formation and maintenance of lipid microdomains. Lipid rafts are important signaling platforms whose structure is sensitive to membrane lipid composition (8), as are the proteins that interact with these and other membrane microdomains (9, 10). Therefore, modifications in SM content affect lipid raft associated signaling pathways. SM is synthesized by SM synthase (SMS) isoforms that catalyze the transfer of a phosphocholine moiety to the primary hydroxyl group of ceramide to form SM and 1,2-diacylglycerol (1,2-DAG) (11). Thus, SMS lies at the crossroads between the two main groups of membrane lipids (glycerophospholipids and sphingolipids) and between two key signaling molecules in cell cycle regulation (ceramide and 1,2-DAG) (12, 13). Consequently, regulating SMS activity will have important consequences on the cell physiology.

In this study, we describe how 2OHOA regulates SMS activity in cancer cell membranes but not in those of nontumor cells, and the concomitant changes in the levels of SM and other lipids. We demonstrate that exposing tumor cells to 2OHOA promotes a robust increase in SM mass through the rapid and specific activation of SMS isoforms. In addition, studies in model membranes showed that these marked changes in lipid composition caused by 2OHOA affect the biophysical properties of membrane microdomains. The rapid activation of SMS by 2OHOA and the subsequent accumulation of SM can at least in part explain the ability of this compound to trigger cell cycle arrest, cell differentiation, and autophagy or apoptosis in cancer cells. Importantly, this report shows that 2OHOA has differential and specific effects against cancer cells.

Results

We assessed the capacity of 2OHOA to diminish the viability of human U118 glioma cells using the XTT method (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt) and evaluating its effect on DHFR levels, a protein
downregulated in other cancer cells treated with 2OHOA (1). In this context, 2OHOA reduced the viability of human U118 glioma cells in a concentration-dependent manner (Fig. 1A). 2OHOA (200 μM, 48 h) also induced a marked and significant decrease in the DHFR protein levels (69.7%) in U118 cells, whereas it had no such effect in the nontumor human lung fibroblast (MRC-5) cells (Fig. 1B). These results extend previous studies, showing that 2OHOA has a distinct effect on DHFR in tumor and nontumor cells (1, 3, 4).

Then, we investigated the impact of 2OHOA treatment (200 μM, 72 h) on U118 cells lipid mass. Thus, treatment with 2OHOA induced a marked and significant increase in SM mass (4.6-fold) (Table 1 and Fig. 2A) and a decrease in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) mass (57 and 30%, respectively). In addition, phosphatidylserine (PS) mass also decreased 43%, whereas phosphatidylinositol (PI) mass remained unchanged. The total phospholipid content and the cholesterol/PL ratio were essentially unaffected by 2OHOA treatment, despite the modest increase in cholesterol (20%) detected in treated cells. In addition, the analysis showed that SM levels increased in a time- and concentration-dependent manner (SI Materials and Methods).

We further analyzed the effect of 2OHOA on the phospholipid composition of human leukemia (Jurkat) cells, nonsmall lung cancer (A549) cells, additional human glioma cell lines (1321N1 and SF767) cells, and in nontumor (MRC-5) cells. Exposure to 2OHOA (200 μM, 24 h) significantly increased the SM content of cancer cells (2.4-fold in Jurkat, 2.7-fold in A549, 2.2-fold in 1321N1, and 36.0% in SF767 cells) but not that of MRC-5 cells (Fig. 2A). These data not only revealed the ability of 2OHOA to regulate the phospholipid composition of tumor cells, but also they indicated that this effect was specific to cancer cells.

Table 1. Changes in phospholipid mass in control and treated cells

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>2OHOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>42</td>
<td>193</td>
</tr>
<tr>
<td>PC</td>
<td>249</td>
<td>176</td>
</tr>
<tr>
<td>PS</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>PI</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>PE</td>
<td>106</td>
<td>46</td>
</tr>
<tr>
<td>Total PL</td>
<td>463</td>
<td>486</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>172</td>
<td>205</td>
</tr>
<tr>
<td>Cholesterol/PL</td>
<td>0.38</td>
<td>0.42</td>
</tr>
</tbody>
</table>

We used a number of analogues (200 μM, 24 h) to investigate the structural bases underlying the regulatory effects of 2OHOA on SM focusing on the fatty acid length, the presence and number of double bonds, and the substitution at C-2 (Fig. 2B). The failure of oleic acid (18:1n-9) and 2-methyl oleic acid (2Me-18:1n-9) to significantly affect SM levels indicated that the presence of the hydroxyp group at C-2 is crucial. In addition, SM levels only increased when cells were treated with 18-C fatty acids containing at least one double bond. Therefore, SM accumulation induced by 2OHOA appears to be strictly related to the structure of the compounds.

Because of the rapid increase in SM, in addition to the decrease in PC content (Table 1), we assessed the SMS activity in the presence of 2OHOA. SMS activity was 3.6-fold higher than in untreated cells after 24 h of treatment (200 μM) (Fig. 3A). Importantly, SMS activity increased 85% after 5 min of treatment, indicating that 2OHOA has an extremely rapid effect on SMS. To demonstrate that 2OHOA directly interacts with SMS, a cell postnuclear supernatant was incubated with NBD-C6-Cer (0.1 μg/μL) and 2OHOA (200 μM) for 2 h. In these in vitro conditions, 2OHOA increased SMS activity 80% (Fig. 3B), indicating a direct interaction between the drug and the enzyme.

To demonstrate the importance of SMS activation in 2OHOA’s effects against tumors, we examined if SMS inhibition affected cell cycle progression. Thus, when A549 cells were exposed to 2OHOA (200 μM, 16 h) in the presence of the SMS inhibitor D609 (350 μM) (14, 15), the effect of 2OHOA on SM levels was reversed from 1.7-fold increase in the absence of D609 to 1.2-fold in its presence, indicating that 2OHOA was not able to exert its effect on SMS (Fig. 4A). Accordingly, the distribution of cells in the G1 phase of the cell cycle shifted from 50 ± 5% in the absence of D609 to 40 ± 4.1% in its presence (values compared to control 31 ± 1.4%; Fig. 4B), showing that SMS inhibition diminishes 2OHOA capability to induce cell cycle arrest.

The specific SM binding protein, lysenin (16), showed that the newly generated SM accumulated mainly at the plasma membrane (Fig. 5A). To understand the consequences that the dramatic changes in phospholipid composition provoked by 2OHOA on the structure and biophysical properties of the plasma membrane, we prepared model membranes with compositions mimicking the major alterations observed (Table 1). These membranes were labeled with one of two membrane probes: diphenylhexatriene (DPH), a fluorophore that shows no preference for liquid ordered or liquid disordered phases, or 1-PnA (trans-parinaric acid), which preferentially incorporates into liquid disordered membrane domains (17).

The increase in DPH steady-state fluorescence anisotropy (DPH (r)) in liposomes mimicking treated cells (Fig. 5B) was indicative of an increase in the general lipid order as this parameter reflects the global order of the acyl chains within the lipid bilayer membrane (9). Because the molar cholesterol fraction was...
similar in treated and control cells (Table 1), the increase in the lipid bilayer order was probably related to the marked increase in SM content. In addition, the changes in phospholipid composition induced an increase in the η-PnA long lifetime component (τ_long), indicating that the ordered domains became more ordered and more compact (Fig. 5C).

**Discussion**

From the molecular point of view, 2OHOA is a first-in-class anticancer lipid because its mechanism of action is not shared by other drugs used to treat cancer. However, its mechanism of action is yet to be fully elucidated, especially with respect to the first of the series of molecular events triggered by this molecule. In the present study, we demonstrate that 2OHOA activates SMS isozymes, inducing a rapid increase in the membrane levels of SM. In addition, this effect appears to be strongly associated with its structure, as only C-18 fatty acids with one or more double bonds and a hydroxyl moiety at C-2 induce SM accumulation.

Our aim was to investigate the effects of 2OHOA on the composition and structure of noncancer and cancer cell membranes, and the relationship of the changes induced with its activity against tumors. In the present work, we demonstrated that 2OHOA induces marked changes in the lipid composition of glioma cells by increasing SM mass in a time- and concentration-dependent manner (Table 1). Similar changes were observed in other glioma, leukemia, and lung cancer cell lines (Jurkat, 1321N1, SF767, and A549 cells), whereas no such differences in phospholipid composition were observed in nontumor MRC-5 cell membranes (Fig. 2A). The incapacity to produce relevant effects on nontumor MRC-5 cells could be due to the fact that these cells already contain high levels of SM, such that SMS activation cannot further increase the amount of this lipid in membranes. Accordingly, lower SM levels in different human tumor tissues when compared to normal tissues (colon, breast, leukemia, esophagus, and brain) have been already described (18). Thus, the markedly lower levels of SM in cancer cells suggest that the malignant transformation of glioma and other cancer cells not only requires the activation of certain oncogenes (e.g., Ras) but also, the modification of the membrane structure to permit their membrane docking and proliferative signal propagation (see below).

The 2OHOA compound augments both SMS1 and SMS2 activities, whereas it has no effect on SMS mRNA and protein levels. Interestingly, activation of SMS by 2OHOA turned out to be extremely rapid and sustained, probably due to the direct interaction between 2OHOA and SMS (Fig. 1B). This direct interaction is likely to explain the structural relationship between the fatty acid structure and SMS activation (Fig. 1B). Indeed, we found that (i) the 2-hydroxy moiety, (ii) 18 C atoms, and (iii) at least one double bond in the fatty acid structure were crucial to induce SM accumulation. An important question was the source of ceramides needed to sustain the large increase in SM. In this case, HPLC analysis showed that sphingosine mass increased 5.4-fold in cells exposed to 2OHOA (200 μM, 48 h), suggesting the activation of at least the salvage pathway as sphingosine is only generated through this route (19).

In model membranes that mimic the lipid composition of U118 cell membranes before and after treatment with 2OHOA, a slight increase in the fraction of l_o domains, but a rather marked rise in the membrane hydrophobic core packing, was observed.
interactions of Ras with upstream (e.g., EGFR) and downstream
insertion into the membrane, which is necessary for subsequent
with loose surface packing (as PE-rich domains) to permit its
has a bulky isoprenyl lipid anchor and requires
and cell cycle arrest in human glioma and lung cancer cells. Ras
the induction of apoptosis observed in Jurkat cells (4).
and structure of membranes induced by 2OHOA also explain
SM synthesis (20), such that the changes in the composition
haptosis pathway (4). Moreover, FasR capping has been linked to
induces Fas receptor (FasR) capping favoring spontaneous inter-
by increases in membrane SM. Thus, in leukemia cells, 2OHOA
as well as increased apoptosis (4). In this context, two different molecular mechanisms are known
to be activated by 2OHOA in cancer cells that may be triggered
in membrane SM. Thus, in leukemia cells, 2OHOA
induces Fas receptor (FasR) capping favoring spontaneous inter-
actions of FasR subunits and the activation of the extrinsic apo-
tosis pathway (4). Moreover, FasR capping has been linked to SM synthesis (20), such that the changes in the composition and structure of membranes induced by 2OHOA also explain the induction of apoptosis observed in Jurkat cells (4).

On the other hand, 2OHOA may induce translocation of Ras and cell cycle arrest in human glioma and lung cancer cells. Ras has a bulky isoprenyl lipid anchor and requires $l_d$ microdomains
with loose surface packing (as PE-rich domains) to permit its
insertion into the membrane, which is necessary for subsequent interactions of Ras with upstream (e.g., EGFR) and downstream (e.g., Raf) proteins in the MAPK pathway. Therefore, the increase in SM diminishes the $l_d$ domains and augments the $l_o$ regions, the net result being an inactivation of the MAPK pathway. Through cross-talk, the inhibition of this pathway can impair the PI3K/Akt pathway and consequently the cell cycle machinery (cyclin/cdk complexes). In addition, increases in 1,2-DAG could be involved in the relatively sustained and mild activation of PKC (about 3-fold) (3). This activation could lead to the overexpression of the CDK inhibitors, p21$^{Cip1}$ and p27$^{Kip1}$, which are associated with the hypophosphorylation of the retinoblastoma protein and the ensuing inhibition of E2F-1 and $\beta$-catenin (2, 3). The cellular outcome of these molecular processes is the inhibition of cell growth, and the induction of cell differentiation and autophagy (1, 21, 22).

Some studies show that SMS inhibition and the ensuing ceramide accumulation are associated with cell cycle arrest (23) or cell death (24, 25). However, reduced SM synthesis causes resistance to certain apoptosis inducing drugs (26, 27). In addition, increased SM membrane content enhanced Fas- and TNF-α-induced apoptosis through an efficient clustering of FasR or by increasing TNF-α receptor exposure at the plasma membrane (20, 26). Analogously, decreased SM levels protected THP-1 derived macrophages from lipopolysaccharide-induced apoptosis, an effect linked to a decrease in cell surface TLR-4 expression (26). For all these reasons, the physiological significance of

![Fig. 4. Inhibition of SMS by D609 diminishes the effect of 2OHOA on cell cycle progression. (A) Effect of SMS inhibition on cell cycle progression. Bar graphs show the percentage of A549 cells in Sub G1, G1, G2, and G2/M phases with respect to the total cell number. (C) Effect of SMS inhibition on SM content. Values represent the mean ± SEM; n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001. In this case, § indicates a significant effect of the inhibition as compared with the treatment (§§ P < 0.01).](image1)

![Fig. 5. Changes in phospholipid composition induced by 2OHOA increase the global order of model membranes. (A) Representative confocal sections of U118 cells labeled with lysenin showing increased SM plasma membrane levels after 2OHOA treatment (200 μM, 72 h). Cells were fixed and incubated with lysenin as described in Materials and Methods). Scale bar, 10 μm. (B) The 2OHOA compound induces an increase in the global order of the membrane. Steady-state fluorescence anisotropy, r, of DPH in PC:PE:SM:Cholesterol mixtures at 24 °C. (C) Treatment induces an increased packing of the $l_o$ domains. Long lifetime component of the mean fluorescent lifetime r$_\text{avg.}$ of t-Pna in POPC:POPE:PSM:Cholesterol mixtures at 24 °C. MM, model membrane. Values represent the mean of triplicates ± SD; n = 3; ** P < 0.01; *** P < 0.001.](image2)
Lipids. The 2OHOA compound (Good Manufacturing Practice quality) was obtained from Avanti Polar Lipids, and its purity was assessed as described previously (1). The 2OH-18:0 and 2Me-18:1n-9 were kindly provided by Oliver Vögler (Department of Biology, University of the Balearic Islands, Palma, Spain). Oleic acid was purchased from Sigma Chemical Company. NBD-C6-Cer and the t-PnA and DPH probes were purchased from Invitrogen, Molecular Probes.

Lipid Analysis. The cellular lipids were extracted directly from the frozen monolayer of cells by a modified n-hexane:2-propanol (3:2, vol/vol) extraction (23, 30). Protein levels were measured using the bicinchoninic acid assay according to the manufacturer’s instructions (Thermo Scientific). Individual phospholipids and neutral lipids were separated by TLC or by high-performance TLC (HPTLC) on Whatman silica gel-60 plates (55:35:3:2 by volume). For TLC, the phospholipid mass was determined by assaying the chloroform/methanol/acidic water (55:37.5:3:2 by volume). Protein levels were measured using the bicinchoninic assay according to the manufacturer’s instructions (29, 30). Protein levels were measured using the bicinchoninic assay according to the manufacturer’s instructions (29, 30). Protein levels were measured using the bicinchoninic assay according to the manufacturer’s instructions (29, 30).

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In Vitro SMS Activity Assay. [Adapted from Villani et al. (26) and Ding et al. (32).] U118 cells were homogenized in ice-cold lysis buffer (50 mM Tris/HCl, 1 mM EDTA, 1 mM PMSF, 8% CHAPS (wt/vol), pH 7.4) by passing 20 times through a 28-gauge needle. The cell lysate was centrifuged at 500 × g for 5 min at 4 °C, and the enzymatic activity was measured in the postnuclear supernatant (100 μg of protein). The substrate was a mixture of 0.1 μg/μL of NBD-C6-Cer, 0.01 μg/μL of synthetic PC and PE reconstituted by sonication and vortexing until it was clear in 50 mM Tris/HCl, 25 mM KCl and 1 mM EDTA, pH 7.4. The substrate was diluted 1:1 with the proteins reconstituted in reaction buffer. The reaction was stopped on ice by addition of 2 vol of chloroform/methanol (1:2, vol/vol) after the incubation period (2 h in dark).

Inhibition Experiments. A549 cells were incubated with D609 for 16 h (350 μM, a gift from A. Llebaria, Department of Biomedical Chemistry, Institut d’Investigacions Químiques i Ambientals de Barcelona–Centro Superior de Investigaciones Científicas, Barcelona, Spain), and 2OHOA (200 μM) was added 1 h after the addition of D609. The effect on the cell cycle was evaluated by flow cytometry. After trypsinization, A549 cells were fixed with ice-cold 70% ethanol for 1 h at 4 °C, centrifuged for 5 min at 1,250 × g, and resuspended in a sodium citrate (38 mM, pH 7.4). Then, cells were incubated for 20 min at 37 °C with buffer A [sodium citrate (38 mM, pH 7.4), 50 μg/mL propridium iodide, and 5 μg/mL RNase A (Sigma-Aldrich)] and analyzed on a Beckman Coulter Epics XL flow cytometer. Cell populations in the different phases of cell cycle (subG1, G0/G1, S, and G2/M) were determined on the basis of their DNA content.

Immunofluorescent Labeling of SM by Lysenin. Cells were grown on Chambered Covergass (Lab-Tek™ II, Thermo Fisher Scientific Inc.) in the presence or absence of 2OHOA (200 μM, 72 h). SM was labeled with lysenin as described previously (16) with minor modifications (see SI Materials and Methods). Images were acquired on a Leica TCS SP2 spectral confocal microscope at 630× optical magnification and 3× digital magnification. The images were analyzed with the software provided by the manufacturer.

Liposome Preparation and Fluorescence Measurements. Multilamellar vesicles (MLV) of POPC, POPE, PSM, and cholesterol mixtures were prepared with neither t-PnA or DPH, or with no probe. MLV suspensions were prepared as previously (10) (molar proportions of each lipid is given in SI Materials and Methods). The probe/lipid ratios used were 1:200 for DPH and 1:500 for t-PnA. Fluorescence measurements were obtained with a Horiba Jobin Yvon FL-1057 Tau 3 spectrophotofluorometer, carrying out the experiments at 24 °C.

Statistics. Statistical analysis was performed using GraphPad Prism 4.01 (GraphPad Software Inc.). Unless specified, the data are expressed as the mean ± SEM, at least three independent experiments, the value represented by n. The statistical significance of the mean difference was determined by Student’s t test. The asterisks indicate a significant effect of the treatment when compared with the control: * P < 0.05; ** P < 0.01; *** P < 0.001.

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