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The role of membrane fatty acid remodeling in the antitumor mechanism of action of 2-hydroxyoleic acid

Maria Laura Martin [a,1], Gwendolyn Barceló-Coblijn [a,1], Rodrigo F.M. de Almeida [b], Maria Antònia Noguera-Salvà [a], Silvia Terés [a], Mónica Higuera [a], Gerhard Liebisch [c], Gerd Schmitz [c], Xavier Busquets [a], Pablo V. Escribá [a,*]

[a] Laboratory of Molecular Cell Biomedicine, Department of Biology, Institut Universitari d'Investigació en Ciències de la Salut, University of the Balearic Islands, Ctra. de Valldemossa Km 7.5, E-07122 Palma, Balearic Islands, Spain
[b] Centro de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Ed. C8, 1749-016 Lisboa, Portugal
[c] Institute for Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany

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ABSTRACT

The synthetic fatty acid 2-hydroxyoleic acid (2OHOA) is a potent antitumor drug that we rationally designed to regulate the membrane lipid composition and structure. The lipid modifications caused by 2OHOA treatments induce important signaling changes that end up with cell death (Terés et al., 2012 [1]). One of these regulatory effects is restoration of sphingomyelin levels, which are markedly lower in cancer cells compared to normal cells (Barceló-Coblijn et al., 2011 [2]). In this study, we report another important regulatory effect of 2OHOA on cancer cell membrane composition: a large increase in 2OHOA levels, accounting for ~15% of the fatty acids present in membrane phospholipids, in human glioma (SF767 and U118) and lung cancer (A549) cells. Concomitantly, we observed marked reductions in oleic acid levels and inhibition of stearoyl-CoA desaturase. The impact of these changes on the biophysical properties of the lipid bilayer was evaluated in liposomes reconstituted from cancer cell membrane lipid extracts. Thus, 2OHOA increased the packing of ordered domains and decreased the global order of the membrane. The present results further support and extend the knowledge about the mechanism of action for 2OHOA, based on the regulation of the membrane lipid composition and structure and subsequent modulation of membrane protein-associated signaling.

1. Introduction

In previous studies, we showed that 2-hydroxyoleic acid (2OHOA, Minerval) exerts its anti-cancer effects by inducing first cell cycle arrest [3–5], followed by apoptosis in leukemia cells [6] or differentiation and autophagy in glioma cells [1]. In this context, 2OHOA is a lipid that binds to the bilayer altering its structure and microdomain properties and distribution [2,5]. Currently, 2OHOA is the first lipid drug rationally designed to target lipid membranes with the aim to interact with them and regulate the membrane lipid composition and structure. The specificity and efficacy of 2OHOA has been recently acknowledged by the European Medicines Agency which has designated 2OHOA as an orphan medicinal product for the treatment of glioma due to its high efficacy and lack of toxicity [7].

We designed 2OHOA to reproduce the antitumor effect of anthracyclines via interactions with the plasma membrane and the consequent modifications in cell signaling [8,9]. One of the events involved in the mechanism of action of 2OHOA is the rapid and sustained activation of sphingomyelin synthase (SMS), being the sphingomyelin (SM) produced predominantly accumulated at the plasma membrane [2]. Despite the importance of the plasma membrane in its anti-cancer effects, the molecular mechanisms underlying the cellular effects of 2OHOA on cancer cells are not fully understood.

This study was designed to investigate the effect of 2OHOA treatments on the fatty acid composition and structure of cancer cell membranes. Exogenously added fatty acids can be incorporated into glycerolipids, either by acylation of glycerophosphate to phosphatidic acid (via the Kennedy pathway), or by remodeling of de novo synthesized glycerophospholipids via deacylation–reacylation or via the monoacylglycerol-pathway in the case of glycerolipids. Although well established for regular fatty acids (i.e., non-hydroxylated fatty acids), the incorporation and metabolism of exogenous 2-hydroxy fatty acids like 2OHOA remains poorly understood [10]. We found

Abbreviations: 2OHOA, 2-hydroxyoleic acid; DAG, diacylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; FAME, fatty acid methyl ester; HPTLC, high performance TLC; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SCD1, stearoyl-CoA desaturase-1; SM, sphingomyelin; SMS, sphingomyelin synthase; TAG, triacylglycerol; *t*-PnA, *trans*-parinaric acid

* Corresponding author at: Laboratory of Molecular Cell Biomedicine, Department of Biology, University of the Balearic Islands, Ctra. de Valldemossa Km 7.5, E-07122 Palma, Balearic Islands, Spain. Tel.: +34 971173331; fax: +34 971173184.
E-mail address: pablo.escriba@uib.es (P.V. Escribá).
[1] Both authors contributed equally to this work.

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that 2OHOA treatments caused a dramatic fatty acid profile remodeling in tumor cells. The most important changes were 2OHOA incorporation into different glycerolipids and a decrease in oleic acid levels, accompanied by an increase in stearic acid levels, which was associated with inhibition of stearoyl-CoA desaturase-1 (SCD1) activity.

Finally, the impact of these changes on the biophysical properties of model membranes was also investigated here, revealing that while the global order of the membrane decreased, the ordered domains became more ordered and compact. Taken together, these findings provide new insight into the mechanism of action of 2OHOA, demonstrating the effects of this compound on the fatty acid composition and structure of lipid bilayer.

2. Materials and methods

2.1. Cell culture

Human glioma cells (U118 and SF767), human non-small lung cancer cells (A549) and MRC5 human fibroblast were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained as described previously [3].

2.2. Lipids

2OHOA (GMP quality) was obtained from Lipopharma and its purity was determined as described previously [3]. All synthetic lipids used in this study were obtained as described previously [2]. [9, 10-^{3}H]-2-hydroxy oleic acid ([^{3}H]-2OHOA) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA).

2.3. Lipid analysis

After extraction with n-hexane:2-propanol (3:2, by vol) [11,12]. The individual phospholipid classes were separated by TLC as described previously [2,13,14]. The phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions were subjected to base-catalyzed transesterification, converting the acyl chains of the phospholipids to fatty acid methyl esters (FAMEs, [15]). Individual FAMEs were separated by gas liquid chromatography using a SP-2330 column (0.32 mm ID, 30 m length: Supelco, Bellefonte, PA, USA) and a gas chromatograph (GC5890 Agilent, USA) equipped with dual autosamplers and dual flame ionization detectors. A 17:0 fatty acid was used as the internal standard.

Neutral lipids were separated in petroleum ether/diethyl ether/acetic acid (75:25:1.3 by vol) [14] and the lipid fractions were identified using authentic standards (Larodan, Sweden). After development, the plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180 °C for 10 min [13]. Lipids were then quantified by photodensitometry and expressed per mg of protein. Protein levels were measured using the bicinchoninic assay, according to manufacturer's instructions (Thermo Scientific, Rockford, USA).

2.4. Determination of SCD-1 activity

The SCD-1 activity assay was adapted from Du et al. and Scaglia et al. [16,17]. Control and treated U118 cells were steady-state labeled for 6 h with trace amounts of [^{3}H]-palmitic acid (0.25 μCi/60 mm cell culture dish; stock at 1 mCi). At the end of the incubation, total cell lipids were extracted and transesterified as described above. The derived methyl esters were separated by argentation TLC following the procedure described by Wilson and Sargent [18]. Lipid fractions were identified using pure methyl stearic acid and methyl oleic acid as standards (Larodan, Sweden). SFA and MUFA spots were scraped and the radioactivity incorporated was quantified by liquid scintillation counting. The level of [^{3}H]-MUFA produced was normalized to cellular protein content.

2.5. Mass spectrometry

Lipid extraction and mass spectrometry based targeted lipid analysis was performed as described previously [19–21]. Briefly, cell pellets were lysed in 0.1% SDS, sonicated and aliquots corresponding to 100 μg of total protein (BCA assay) were used for lipid extraction. Direct flow injection was performed with a 1200 series binary pump (Agilent, Waldbronn, Germany) coupled to a Quattro Ultima tandem mass spectrometer (Micromass, Manchester, UK) via electrospray ionization (ESI). Reversed phase and HILIC LC–ESI-MS/MS was performed using a 1200 series binary pump and a hybrid triple quadrupole linear ion trap mass spectrometer API 4000 Q-Trap (Applied Biosystems, Darmstadt, Germany). Fatty acid species were analyzed after FAME derivatization using a Shimadzu 2010 GC–MS, quantifying fatty acids by calibrating with the standards of naturally occurring lipid species added to the cell homogenates or plasma. The following compounds were used as internal standards were: PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0 (di-phytanoyl), PS 14:0/14:0, PS 20:0/20:0 (di-phytanoyl), PG 14:0/14:0, PG 20:0/20:0 (di-phytanoyl), PI 17:0/17:0, LPC 13:0, LPC 19:0, Cer 14:0, Cer 17:0, D7-FC, CE 17:0 and CE 22:0. The calibration lines used for quantification were generated in the matrix with the following species: PC 34:1, 36:2, 38:4, 40:0 and PC O 16:0/20:4; SM 16:0, 18:1, 18:0; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6 and PE p16:0/20:4; PS 34:1, 36:2, 38:4, 40:6; Cer 16:0, 18:0, 20:0, 24:1, 24:0; FC, CE 16:0,18:2,18:1,18:0.

2.6. Incorporation of [^{3}H]-2OHOA in MRC-5 and U118 cells

MRC-5 and U118 cells were pulse labeled for 1, 5, 15, 30 min, 1 and 2 h with trace amounts of [^{3}H]-2OHOA (0.25 μCi/60 mm cell culture dish; stock at 1 mCi). After the labeling period, cells were thoroughly washed for three times with ice-cold PBS. Cell homogenates were transferred into scintillation tubes and the radioactivity was measured in a scintillation counter (Beckman, LS-6500).

2.7. Liposome preparation from lipid extracts

Lipid extracts were dissolved in chloroform/methanol (2:1) to obtain a concentration of 1 μmol Pi/ml. Total lipid concentration in the MLV suspensions was 0.2 mM in every sample, and the medium used for suspension was sodium phosphate 10 mM, NaCl 150 mM, EDTA 0.1 mM buffer, pH 7.4 and multilamellar vesicles (MLVs) were prepared as described previously [22,23] and equilibrated overnight in darkness.

2.8. Artificial liposome preparation and addition of 2OHOA

Appropriate volumes of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, N-palmitoyl-sphingomyelin, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and cholesterol stock solutions in an organic solvent were mixed to obtain the required molar lipid ratios (Table S1) [2]. MLV were prepared as described above, without a probe and at final lipid concentrations of 0.5 mM to ensure efficient incorporation of 2OHOA into the lipid bilayer. To obtain LUV, MLV suspensions were extruded using an Avanti Mini-Extruder and polycarbonate filters (100 nm pore diameter: Nuclepore, Whatman). Different aliquots of LUV suspension were labeled with either *t*-PnA or DPH added from stock ethanol solution, and incubated for 1 h at 50 °C [24]. The suspension was slowly brought to room temperature and allowed to equilibrate before 2OHOA was added at a final concentration of 25 μM (5 mol%) or 100 μM (20 mol%) at least 1 h before the fluorescence was measured (all samples were stored in the dark). The 2OHOA/lipid ratio in the 25 μM 2OHOA samples is similar to the estimated 2OHOA/lipid ratio in cells treated with 200 μM of the drug.

2.9. Fluorescence measurements and data analysis

Fluorescence was measured at 24 °C using a Horiba Jobin Yvon FL-1057 Tau 3 spectrofluorometer as described previously [2].

2.10. Statistics

Statistical analysis was performed using GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated the data are expressed as the mean ± SEM from at least three independent experiments (n). The statistical significance of the mean difference was determined by the Student's *t* test. Asterisks indicate a significant effect of the treatment as compared with controls (*P<0.05; **P<0.01; ***P<0.001).

3. Results

3.1. 2OHOA reduces oleic acid content in tumor cells

We recently demonstrated that tumor cell phospholipid composition is significantly modified by exposure to 2OHOA [2] and thus, we analyzed here the effects of 2OHOA on tumor cell fatty acid composition. Human glioma (U118) cells were treated with 2OHOA (200 μM, 72 h) and after lipid extraction, their PE and PC fractions were collected from TLC plates, transesterified in basic conditions and analyzed by gas–liquid chromatography. Exposure to 2OHOA profoundly affected the PE and PC fatty acid composition (Table 1) and notably, oleic acid (18:1n−9), which is structurally related to 2OHOA, was reduced by 43% in the PC and 60% in the PE fractions. This decrease, along with a reduction in *cis*-vaccenic acid (18:1n−7, decreased 23% and 40% in PC and PE, respectively), accounted for most of the total MUFAs lost (39% in PC and 53.5% in PE). Conversely, SFA levels increased by 45% in the PC and 53.5% in the PE fractions. Palmitic acid (16:0) and stearic acid (18:0) SFAs increased by 40% and 58% in the PC, respectively, while in the PE fraction, palmitic acid and stearic acid SFA increased by 2.5-fold and 76%, respectively. In addition, total PUFA levels increased 2.5-fold in the PC fraction while no significant change was observed in the PE fraction. Similar changes were obtained when data were expressed in mass (nmol FAME/mg prot, Table S2).

Table 1
Effect of 2OHOA on the fatty acid composition of individual phospholipids in human glioma U118 cells.

FAME	Phosphatidylcholine (PC)		Phosphatidylethanolamine (PE)	
	Control	2OHOA	Control	2OHOA
16:0	31.2 ± 0.6	43.8 ± 1.2***	4.4 ± 0.4	10.8 ± 0.1***
16:1	3.2 ± 0.1	1.3 ± 0.1***	0.2 ± 0.1	0.9 ± 0.1**
18:0	7.0 ± 0.1	11.1 ± 0.2***	15.4 ± 0.2	27.1 ± 0.2***
18:1n−9	40.8 ± 0.3	23.4 ± 0.5***	33.2 ± 0.9	13.3 ± 0.1***
18:1n−7	12.5 ± 0.2	9.7 ± 0.3***	9.5 ± 0.2	5.7 ± 0.1***
18:2n−6	0.8 ± 0.0	1.5 ± 0.0***	ND	ND
20:1n−9	1.1 ± 0.1	0.6 ± 0.2*	ND	ND
20:3n−6	0.5 ± 0.1	1.1 ± 0.1***	2.7 ± 0.2	2.4 ± 0.1
22:0	0.8 ± 0.0	1.7 ± 0.1***	3.5 ± 0.4	6.3 ± 0.4**
20:4n−6	0.8 ± 0.1	1.6 ± 0.1***	12.6 ± 0.1	8.7 ± 0.1***
20:5n−3	0.3 ± 0.1	0.4 ± 0.0	2.9 ± 0.2	1.3 ± 0.1***
22:5n−3	0.5 ± 0.1	1.8 ± 0.2***	8.5 ± 0.3	11.6 ± 0.1***
22:6n−3	0.6 ± 0.1	2.1 ± 0.4**	7.1 ± 0.8	11.8 ± 0.2***
SFA	39.0 ± 0.5	56.6 ± 0.9***	23.3 ± 0.3	44.3 ± 0.4***
MUFA	57.6 ± 0.2	35.0 ± 0.8***	42.9 ± 1.0	19.9 ± 0.2***
PUFA	3.4 ± 0.4	8.4 ± 0.6***	33.9 ± 1.2	35.8 ± 0.6

U118 cells were incubated in the presence or absence of 2OHOA (200 μM, 72 h), and lipids were subsequently extracted and analyzed by TLC. PC and PE fractions were converted to FAME in basic conditions and analyzed by gas chromatography. The values are expressed in mol% and represent the mean ± SEM (n = 4–5). Asterisks indicate significant effects compared with controls (**P<0.01; ***P<0.001).

In addition, we analyzed the effect of 2OHOA treatment on a human non-tumor cell line (MRC-5 cells). The results showed minor significant changes in 18:0 (decreased by 10%) and in two minor fatty acids, 20:3n−6 (decreased by 40%) and 16:1, increased by 2.4-fold. Importantly, no changes in MUFA content were observed in non-tumor cells (MRC5, human fibroblasts) exposed to 2OHOA (Table S4). Because fatty acid remodeling is not a process specific for tumor cells, we investigated if the incorporation of 2OHOA in both tumor (U118) and non-tumor cells (MRC-5) could explain the lack of changes in the latter. In each case the [^{3}H]-2OHOA uptake by these cell types was linear over the studied time frame (Fig. 1, $y = 0.272x + 1.1$, $R^2 = 0.864$ for U118 cells and $y = 0.075x + 1.4$, $R^2 = 0.853$ for MRC-5). Therefore, the results clearly showed that the [^{3}H]-2OHOA uptake was 3.6-fold faster in tumor cells than in non-tumor cells.

To further evaluate the remodeling of the fatty acid profile induced by 2OHOA, we analyzed the PC and PE fatty acid composition in U118 cells treated with 200 μM 2OHOA for 12, 24, 48 and 72 h (Fig. 2). 2OHOA provoked a significant reduction in oleic acid levels in both phospholipid fractions at all the time points analyzed, with a concomitant increase in stearic acid levels. Interestingly, oleic acid levels in control (untreated) cells increased with time (from 31% at 12 h to 42% at 72 h in the PC fraction, and from 20% at 12 h to 33% at 72 h in the PE fraction), while remaining constant in 2OHOA-treated cells (24% in the PC and 14% in the PE fraction). Conversely, while no changes in stearic acid levels were observed over time in control cells (7% in the PC and 14% in the PE fraction), increases were detected following exposure to 2OHOA (from 9% at 12 h to 11% at 72 h in the PC fraction; and from 19% at 12 h to 30% at 72 h in the PE fraction).

To determine whether the changes in fatty acid composition observed were exclusive to U118 cells, we analyzed whether 2OHOA altered the fatty acid composition in human non-small lung cancer (A549) cells and in an additional human glioma cell line (SF767, Fig. 3). Exposure of A549 cells to 2OHOA (200 μM, 72 h) decreased the amount of oleic acid in the PC and PE fractions by 47% and 59%, respectively, while stearic acid levels increased 2.7-fold in the PC and 62% in the PE fractions. Similarly, 2OHOA reduced the oleic acid levels in SF767 cells by 17% and 40% in the PC and PE fractions, respectively, while significant increases in stearic acid levels (19%) were only observed in the PE fraction. Collectively, these results indicate that the effects of 2OHOA on fatty acid composition are not dependent on tumor type.

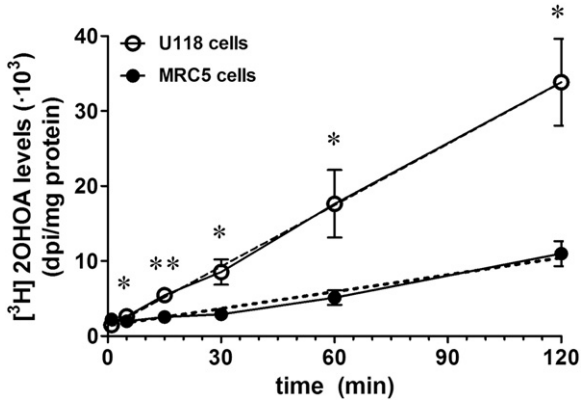

Fig. 1. Differential incorporation of [^{3}H]-2OHOA into U118 compared to MRC-5 cells. U118 (human glioma cells, filled circles) and MRC-5 (human lung fibroblast cells, unfilled circles) were pulse labeled for 1, 5, 15, 30, 60 and 120 min with trace amounts of [^{3}H]-2OHOA (0.25 μCi/60 mm cell culture dish; stock at 1 mCi). Asterisks indicate a significant effect when compared with controls (*P<0.05; **P<0.01).

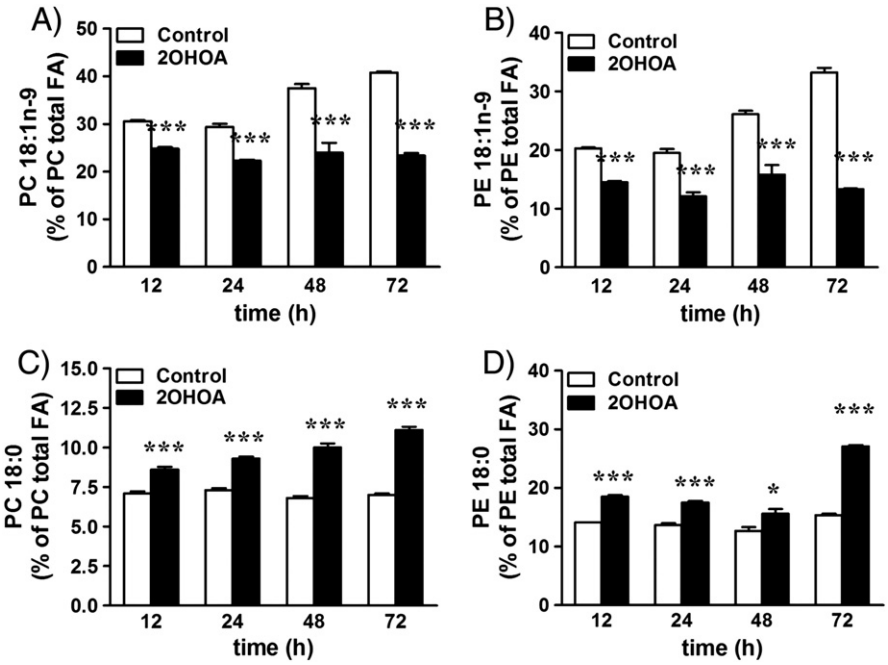

Fig. 2. Time-dependent changes in fatty acid composition of 2OHOA-treated U118 cells. U118 cells were incubated in the presence or absence of 2OHOA (200 μM, 12–72 h) and subsequently, the lipids were extracted and analyzed by TLC. PC (A and C) and PE (B and D) fractions were converted to FAME under basic conditions and analyzed by gas chromatography. Values are expressed as mol% and represent the mean ± SEM (n = 4–5). Bar graphs show oleic acid (A, D) and stearic acid (B, E) content. Asterisks indicate a significant effect when compared with controls (*P<0.05; ***P<0.001).

3.2. 2OHOA inhibits SCD1

The dramatic decrease in oleic acid together with the observed increase in stearic acid (Table 1, Figs. 2 and 3), in both the glycerophospholipid fraction and the total lipid extract (Table S3), strongly suggests that the rate of Δ9-desaturation may be modified by 2OHOA. Hence, we evaluated the effect of 2OHOA on SCD1 activity by measuring the conversion of exogenous [^{3}H]-palmitic acid (16:0) to MUFA. U118 human glioma cells were used in this experiment as no changes were detected either in phospholipid or in fatty acid composition in non-tumor cells after exposure to 2OHOA (Table S4, [2]). Control and 2OHOA-treated cells (200 μM, 48 h) were incubated with [^{3}H]-palmitic acid for 6 h prior to lipid extraction and the total lipid extract was then transesterified in basic conditions, separating the resulting fatty acid methyl esters (FAME) by argentation TLC. When the radioactivity in the spot corresponding to MUFA was measured by liquid scintillation counting, a 60% decrease in [^{3}H]-MUFA was observed in 2OHOA-treated cells compared to control levels (Fig. 4), indicating

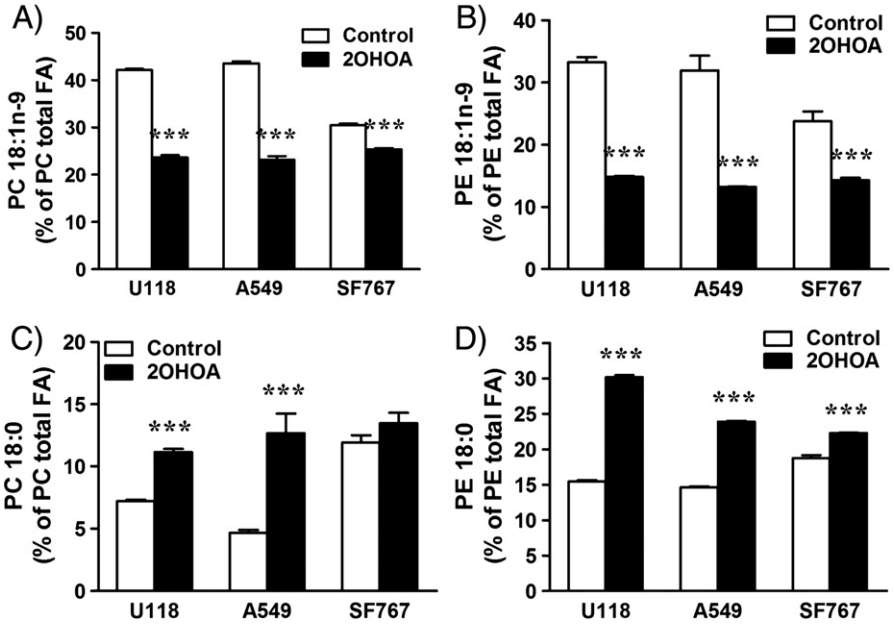

Fig. 3. 2OHOA reduces the oleic acid content of different cancer cell lines. Different cancer cell lines (U118, SF767 and A549) were maintained in the presence or absence of 2OHOA (200 μM, 72 h) and subsequently, the lipids were extracted and separated by TLC. The PC (A and C) and PE (B and D) fractions were converted to FAME under basic conditions and analyzed by gas chromatography. Bar graphs show the oleic acid (A, B) and stearic acid (C, D) content. Values are expressed as mol% and represent the mean ± SEM (n = 4–5). Asterisks indicate a significant effect of 2OHOA compared with controls (***P<0.001).

that SCD1 was inhibited by 2OHOA. Importantly, a similar decrease was observed when cells were exposed to lower (50 and 100 μM) or higher concentrations (300 μM) of 2OHOA. In addition, while oleic acid (150 μM), a known inhibitor of SCD1, reduced [^{3}H]-MUFA levels by 70%, no changes were observed after cell exposure to elaidic acid (200 μM).

3.3. 2OHOA is incorporated into phospholipids

TLC analysis of lipid extracts allowed the detection of free 2OHOA in treated U118 cells (Fig. S1) showing a retention factor (R_f) considerably different to that for non-hydroxylated free fatty acids. Further mass spectrometry (MS) analysis revealed that 2OHOA was incorporated into the glycerolipids triacylglycerol (TAG) and diacylglycerol (DAG) and the glycerophospholipid fractions (Figs. 5 and S2). We further investigated the presence of 2OHOA in lipids by exposing U118 cells to this fatty acid (200 μM) for different periods of time (0.5 to 72 h). We observed a clear time-dependent accumulation of 2OHOA into the glycerophospholipid fraction, with the strongest incorporation into PC (30.1%) and PE (29.0%) after 72 h (Fig. 5). In a similar period 2OHOA was incorporated into phosphatidylserine (PS, 11.7%) and phosphatidylinositol (PI, 13.4%) to a lesser extent. Consistent with our findings in total lipid extracts (Fig. S1), significant amounts of 2OHOA were incorporated into PC after only 0.5 h, whereas similar effects were observed in PE after a 6 h exposure. In PS and PI, significant 2OHOA incorporation was observed after a 24 h exposure. These results indicate that 2OHOA replaces its analog, oleic acid, confirming that the kinetics of fatty acid remodeling depend on the type of glycerophospholipids [25,26].

3.4. 2OHOA induced TAG accumulation

As 2OHOA was also incorporated into TAG (Fig. S2), we used HPTLC to study the effect of 2OHOA on this lipid fraction in extracts from control and 2OHOA-treated (200 μM) U118 cells. Although TAG levels increased following exposure to 2OHOA at all the times studied, these changes followed a cyclic pattern (Fig. 6). Accordingly, the increases in TAG peaked after a 12 h and 48 h exposure (6.7- and 8.5-fold, respectively), while more modest increases were observed after 24 h and 72 h (2.6- and 2.4-fold, respectively). The fatty acid analysis by GC showed that the exposure to 2OHOA affected TAG fatty acid composition (Table 2). Thus, while oleic acid (18:1n−9) and *cis*-vaccenic acid (18:1n−7) accounted for most of the reduction in MUFA levels (ca. 24%), palmitic acid level (16:0) increased by 1.6-fold, accounting for most of the increase in SFA (1.4-fold). Consistently, similar results were obtained when total fatty acids were analyzed (Table S3): oleic acid and *cis*-vaccenic acid were reduced ca. 53%, and 33% respectively, while palmitic acid increased ca. 1.3-fold.

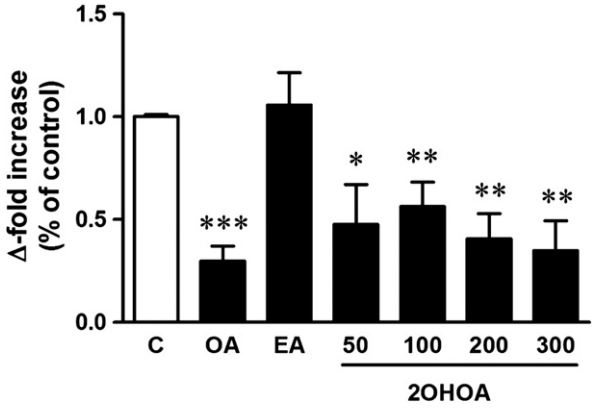

Fig. 4. 2OHOA inhibits SCD1. U118 cells maintained in the presence or absence of 2OHOA (50, 100, 200 and 300 μM), oleic acid (OA, 150 μM) and elaidic acid (EA, 200 μM) for 48 h were labeled for 6 h with [^{3}H]-palmitic acid (0.25 μCi/60 mm Petri dish). Conversion of [^{3}H]-palmitic acid to [^{3}H]-MUFA was measured by following the separation of FAME on TLC plates impregnated with silver nitrate. Radioactivity was measured as indicated in the Materials and methods section. The values represent the mean ± SEM (n = 3–4) and the asterisks indicate significant effects compared with controls (*P<0.05; **P<0.01).

3.5. Effect of 2OHOA on the biophysical properties of cell membranes

We previously reported that exposure to 2OHOA (200 μM, 24 h) increases the lateral packing of ordered domains (l_o) and the global membrane order in artificial liposomes, mimicking the phospholipid composition of cells exposed to this agent [2]. Hence, we assessed the effect of the changes in the phospholipid fatty acid composition induced by 2OHOA on the structural properties of cell membranes by analyzing the biophysical properties of liposomes reconstituted from lipids extracted from untreated and 2OHOA-treated cells. It is worth mentioning that using this system changes in both fatty acid and phospholipid composition were taken into account. To simplify the description of the results, liposomes reconstituted from lipid extracts from control or 2OHOA-treated cells will be referred to as C_L and T_L, respectively. These liposomes were labeled with one of two membrane probes, DPH (diphenyl hexatriene) or *t*-PnA (*trans*-parinaric acid), to provide a broad overview of the changes induced by 2OHOA [27].

DPH is a fluorophore that binds to the lipid bilayer and intercalates parallel to the acyl chains of the phospholipids, showing no preference for liquid disordered (l_d) or liquid ordered (l_o) phases. Thus, the steady-state fluorescence anisotropy of DPH (DPH <r>) reflects the global order of the acyl chains within the lipid bilayer [28,29]. Contrary to our observations in artificial membranes [2], DPH <r> values for T_L were lower than those for C_L at all treatment times, indicating a general decrease in global membrane order (Fig. 7A).

Conversely, *t*-PnA is a fluorophore that is preferentially incorporated into l_o domains, where its fluorescence quantum yield increases [30]. In accordance with our observations in artificial liposomes [2], the changes in lipid composition induced by 2OHOA increased the *t*-PnA long lifetime component (τ_{long}), indicating that the ordered domains became more ordered and more compact (Fig. 7B). Indeed, while the *t*-PnA τ_{long} for C_L was generally below 30 ns, suggesting the presence of sphingolipid–cholesterol enriched domains (lipid rafts), it was always considerably greater than 30 ns in T_L, indicating the possible formation of a sphingolipid-enriched gel-like phase [30]. These results are consistent with the accumulation of SM and other sphingolipids observed in 2OHOA-treated cells [2]. Moreover, the mean fluorescence lifetime of *t*-PnA (τ) paralleled the long lifetime component, indicating that the tighter lipid packing in the l_o domains is the primary factor affecting *t*-PnA fluorescence lifetime (see Fig. S2).

We investigated whether partition of free 2OHOA into membranes could explain the observed differences in DPH <r> between artificial membranes and lipid extracts. Large unilamellar vesicles (LUVs) that mimicked the composition of cells maintained in the presence or absence of 2OHOA for 24 h treatment [2] were prepared with commercial lipids (Table S1) and they were exposed to 2OHOA (5 or 20 mol%) for 1 h (5 mol% represents 200 μM, the concentration of 2OHOA most frequently used in this study). In these experiments, model membranes mimicking the composition of control (C_{MM}) cells that were incubated with 2OHOA (C + 5 or 20 mol% 2OHOA), reflect the initial stages of exposure, when 2OHOA first comes into contact with the cell, while model membranes mimicking the treated cells (T_{MM}) incubated with 2OHOA (5 or 20 mol%), reflect the lipid composition of extracts at the end of the 24 h treatment.

Consistent with our previous findings in artificial liposomes, the DPH <r> of T_{MM} was higher than that of C_{MM} (Fig. 7C) and after incubation with 5 mol% 2OHOA, the DPH <r> only decreased in T_{MM} (T + 5 mol% 2OHOA). This reduction in DPH <r> was more pronounced

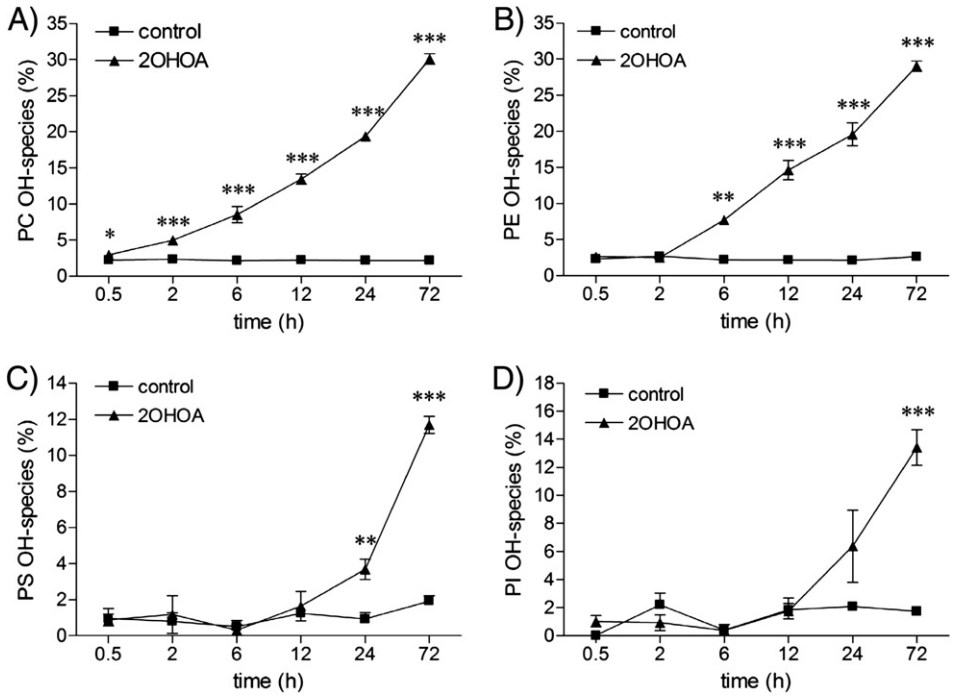

Fig. 5. 2OHOA is partially incorporated into the phospholipid fraction in treated U118 cells. U118 cells were maintained in the presence or absence of 2OHOA (200 μM, 0.5–72 h) and subsequently, their lipids were extracted and analyzed by LC/MS. The values are expressed as the percentage of total fatty acids and represent the mean ± SEM (n = 3–4). Graphs show the content of the OH-species PC (A), PE (B), PS (C) and PI (D) fractions and the asterisks indicate significant effects compared with controls (*P<0.05; **P<0.01; ***P<0.001).

when T_{MM} were exposed to 20 mol% of 2OHOA (T + 20 mol% 2OHOA, Fig. 7C). Hence, the partition of free 2OHOA appears to decrease the global order of model membranes. Nonetheless, the partition of free 2OHOA into membranes only partially explains the effect observed in lipid extracts, in which the decrease in DPH <r> was more pronounced, and this partition did not compensate for the difference in phospholipid composition between control and treated cells (Fig. 7A).

Based on the findings in artificial liposomes [2] and as expected, the *t*-PnA τ_{long} increased in T_{MM} with respect to C_{MM} (Fig. 7D and F, T vs. C). The addition of 5% 2OHOA slightly diminished the *t*-PnA τ_{long} in C_{MM} alone (Fig. 7D), an effect that was enhanced at higher 2OHOA concentrations (20%). However, despite the observed decrease in *t*-PnA τ_{long} in T_{MM} (T vs. T + 20% 2OHOA), 2OHOA failed to counteract the ordering effect induced by the increase in SM (C vs. T + 20% 2OHOA). This decrease in *t*-PnA τ_{long} indicates that 2OHOA was partitioned into both the l_d and l_o domains, as partitioning of 2OHOA into l_d domains only should not affect *t*-PnA τ_{long} (which is only associated with changes in l_o domains). In addition, the mean fluorescence lifetime of *t*-PnA (τ) paralleled the long lifetime component (Fig. S3), indicating that the increased compactness of l_o domains is the main factor affecting the lifetime of *t*-PnA fluorescence, as described above in the C_L and T_L samples. The contrasting *t*-PnA τ_{long} and DPH <r> values also demonstrate the differential effect of 2OHOA on l_o and l_d. As neither changes in phospholipid composition nor the partitioning of free 2OHOA into the membranes can explain the disordering effects of 2OHOA treatment on l_d domains in reconstituted liposomes, we propose that this effect is associated with the changes in fatty composition described above.

4. Discussion

In contrast with most anticancer drugs, 2OHOA is targeted at the plasma membrane, where it regulates the composition and structure of the lipid bilayer. The present study shows the marked remodeling of the fatty acid profile of tumor cells upon exposure to 2OHOA. The relevant regulatory effects exerted by this lipid on the glioma cell

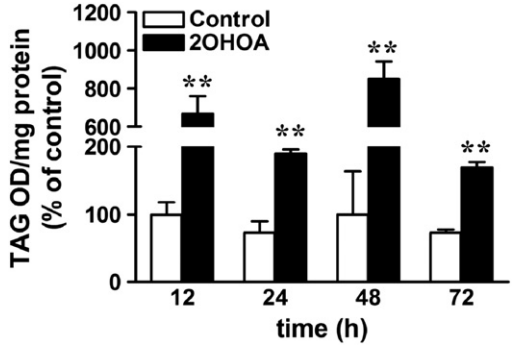

Fig. 6. Effect of 2OHOA treatment on TAG levels in U118 cells. U118 cells were exposed to 2OHOA (200 μM) for different times. Lipids were extracted and neutral lipids were analyzed by HPTLC. Results are means ± SEM, n = 3. The asterisks indicate a significant effect of the treatment as compared with the control (**P<0.01).

Table 2
Effect of 2OHOA on the fatty acid composition of TAG in human glioma U118 cells.

FAME	Control	2OHOA
16:0	16.8 ± 0.7	27.2 ± 1.0***
16:1	1.4 ± 0.9	1.5 ± 0.6
18:0	12.6 ± 1.1	14.7 ± 0.6
18:1n−9	34.1 ± 1.7	24.4 ± 2.6*
18:1n−7	15.9 ± 0.4	11.8 ± 0.6***
18:2n−6	6.3 ± 0.7	2.5 ± 0.7**
20:1n−9	3.3 ± 1.1	2.2 ± 1.0
22:0	9.8 ± 1.3	11.9 ± 0.7
SFA	39.2 ± 1.7	53.8 ± 0.8***
MUFA	54.6 ± 2.2	41.7 ± 1.1***
PUFA	6.3 ± 0.7	4.4 ± 1.6

U118 cells were incubated in the presence or absence of 2OHOA (200 μM, 72 h), and lipids were subsequently extracted and analyzed by TLC. TAG fraction was converted to FAME in basic conditions and analyzed by gas chromatography. The values are expressed in mol% and represent the mean ± SEM (n = 4–5). Asterisks indicate significant effects compared with controls (**P<0.01; ***P<0.001).

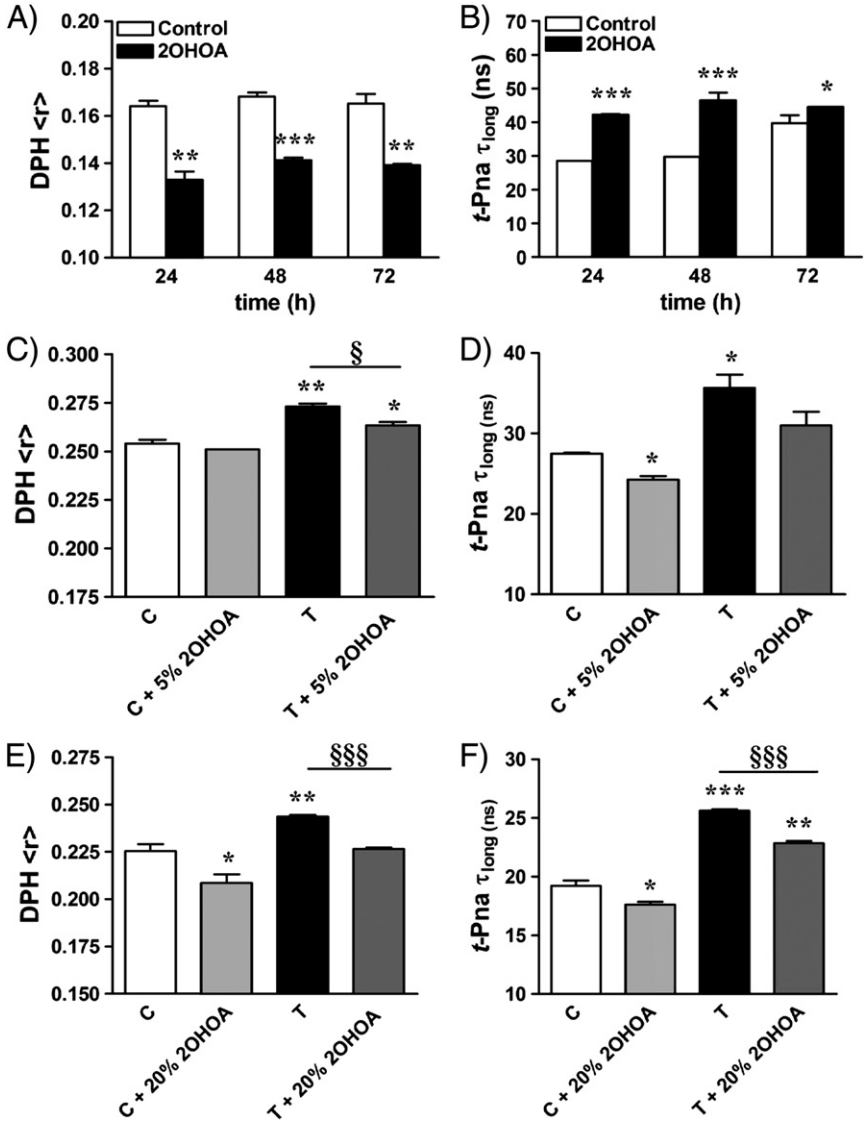

Fig. 7. Biophysical studies to evaluate the role of fatty acid remodeling in the antitumor effects of 2OHOA. Analyses of reconstituted liposomes revealed that 2OHOA induced a decrease in global membrane order, while increasing the order of l_o domains. (A) DPH anisotropy (<r>) and (B) *t*-PnA long lifetime component (τ_{long}) in lipid extracts from control and 2OHOA-treated cells (200 μM; 24, 48 and 72 h) reconstituted into liposomes. Studies in model membranes indicated that the partition of free 2OHOA partially explains its effects on the biophysical properties of the membrane. (C, E) DPH anisotropy (<r>) and (D, F) *t*-PnA long lifetime component (τ_{long}) of LUV mimicking the 24 h lipid composition of control (C) and 2OHOA-treated (T) cells (5 mol%, C and D; or 20 mol%, E and F) at 24 °C. Asterisks indicate significant effects compared with controls (*P<0.05; **P<0.01; ***P<0.001) and § indicates a significant effect of added 2OHOA (T + 2OHOA) as compared with treated cells (T) (§P<0.05; §§§P<0.001). Values represent the mean ± SEM (n = 3).

membrane lipid composition and structure in part explain the previously demonstrated changes in the localization and activity of pivotal signaling proteins (e.g., PKC, Ras) and the concomitant changes in cell signaling that justify the pharmacological effects of 2OHOA against cancer [1,3,5,6]. Oleic acid, a structural analog of 2OHOA, was the natural fatty acid most affected, decreasing by 20–50% in PC and 40–60% in PE in response to 2OHOA treatment, depending on the cell line. This decrease was most likely mediated by the substitution of oleic acid synthesis by 2OHOA and partly to the inhibition of its synthesis, and represents a dramatic effect given that oleic acid is the most abundant fatty acid in these cell lines.

MS analysis of the glycerophospholipid fraction revealed that when U118 cells are exposed to 2OHOA it may account for approximately 15% of their total fatty acid composition [2], becoming one of the most abundant membrane fatty acids in these cells. Interestingly, 2OHOA was also incorporated into the neutral lipid fractions, TAG and DAG, yet not into sphingolipids. This latter finding was somewhat unexpected as endogenous hydroxy fatty acids occur almost exclusively as N-acyl chains within the ceramide moiety of a variety of sphingolipids [31]. This difference may reflect the exogenous nature of 2OHOA, which after conversion to 2OHOA-CoA [10] could be involved in the rapid turnover of the acyl moiety of phospholipids [32,33] or enter the Kennedy-pathway after incorporation into DAG [34]. Taken together, the present findings provide the first evidence of hydroxy fatty acid incorporation into glycerolipids.

In human glioma cells, it has been found that SCD1 activity decreased by ~40–60% upon exposure to 2OHOA, suggesting that SCD1 inhibition may also contribute to the antitumor effect of this fatty acid. A key hallmark of cancer cells is the constitutive activation of fatty acid biosynthesis to sustain the increasing demand for new membrane phospholipids with an appropriate acyl composition. Stearoyl-CoA desaturases are key regulators of such processes [35] and accordingly, SCD1 activity has been strongly associated with membrane lipid synthesis in neoplastic cells [36,37]. Moreover, the induction of apoptosis downregulates SCD1 activity and expression, in conjunction with lower oleic acid and higher stearic acid levels [36,38,39]. Consistently,

2OHOA induces cell cycle arrest in tumor cells, followed by differentiation and autophagy in the case of human glioma cells, or apoptosis in human leukemia cells [1–3,5,6]. The inhibition of SCD1 by 2OHOA may be due to its strong structural similarity to oleic acid, whereby 2OHOA may be recognized by SCD1 as an end product, consequently inhibiting its activity. Alternatively, SCD1 may be inhibited by the oleic acid released when it is substituted by 2OHOA, as oleyl-CoA is a strong competitive inhibitor of the desaturase enzyme [40].

We demonstrated that the profound fatty acid remodeling induced by 2OHOA affects the biophysical properties of cell membranes. In a previous study, we used model membranes to demonstrate that a robust increase in SM augments the global membrane order and concomitantly, the lipid raft order [2]. Here, we used liposomes reconstituted from cell lipid extracts to evaluate how fatty acid remodeling after vehicle or 2OHOA treatments affected the biophysical properties of the membrane. Consistent with our previous results, exposure to 2OHOA increased the l_o domain order, although this effect was accompanied by a decrease in the global order of the membrane, the latter in part explained by the presence of 2OHOA in membranes. In our previous study [2], we could not observe the important decrease in global membrane order shown here. This fact could be due to differences in the fatty acid composition between the model membranes systems used there (commercial synthetic phospholipids) and the reconstituted liposomes used here (from cancer membrane lipids). In that study, the synthetic lipids used mimicked the composition of 2OHOA-treated and -untreated cancer cells in terms of major phospholipid species but there were differences in the type and abundance of fatty acyl moieties and other lipid species present in cell lipid extracts. In addition, another difference between the previous and the present study was the presence of 2OHOA in its free fatty acid form [2] or incorporated into glycerophospholipids, respectively.

The observed changes in membranes levels of SM from 2OHOA-treated cells could account for the increased order of the l_o domains. However, these changes do not explain the increased disorder of l_d domains, which must therefore be due to the alterations in the lipids acyl chain composition of such domains shown in the present study. In agreement with our previous studies showing the effect of hydroxylated glycerophospholipids on the biophysical properties of membranes [41], 2-hydroxylation of the fatty acyl chain of sphingolipids in *Saccharomyces cerevisiae* cells significantly reduces acyl chain packing of their sphingolipid-enriched domains [42]. Thus, the decrease in global membrane order could be attributed to 2OHOA incorporation into the membrane and to the additional changes observed in fatty acid composition, whereas the marked accumulation of SM in membranes of 2OHOA-treated cells could account for the increased packing of l_o domains [2].

The present results explain in part and are in agreement with previous data suggesting a dual-mode mechanism of action for the anticancer drug 2OHOA [1,5]. On the one hand, 2OHOA treatments induce dramatic and selective increases in membrane SM levels only in cancer cells by activating SMS, ultimately increasing the order of l_o domains [2]. This modification to the properties of lipid rafts may contribute to the specific effects already demonstrated of 2OHOA in cancer cells [2], provoking capping of the death receptor FasR and subsequent apoptosis of human leukemia (Jurkat) cells [6] or Ras translocation from the membrane to the cytosol and autophagy of human glioma (SF767) cells [1]. On the other hand, the large incorporation of 2OHOA into phospholipids, and to a lesser degree its membrane binding as free fatty acid, increases the global membrane disorder. Because l_o domains become more compact upon exposure to 2OHOA, a decrease in global order probably reflects a decreased order of l_d domains. This disordering effect, associated with reduction in the surface lateral pressure of the lipid bilayer and the presence of 2OHOA in membranes have previously been shown to induce translocation of PKC to the membrane [1,5,41]. The latter effect was associated with the overexpression of CDK inhibitors, such as $p21^{Cip1}$, the inactivation of different cdks (cdk2, cdk4 and cdk6) and cyclins (A, B, D), and the hypophosphorylation of the retinoblastoma protein, which prevents its dissociation from E2F-1, a pivotal transcription factor in cell cycle progression [4,5]. The final outcome of these molecular processes is cell growth inhibition.

In summary, this is the first report to show the incorporation of 2OHOA in membrane phospholipids, an important aspect of its metabolism in human brain cancer cells. In addition, it has been shown here that treatments with 2OHOA caused a marked remodeling of cancer cell membrane fatty acid composition, in which SCD1 inhibition appears to be a key player. These changes caused an important modulation of the cell membrane microdomain structural features that are associated with regulation of the interaction of peripheral proteins with membranes. Finally, the large changes observed in lipid membranes justify the marked signaling changes that specifically occur in cancer but not normal cells [1].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.01.013.

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