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Changes in Membrane Organization upon Spontaneous Insertion of 2-Hydroxylated Unsaturated Fatty Acids in the Lipid Bilayer

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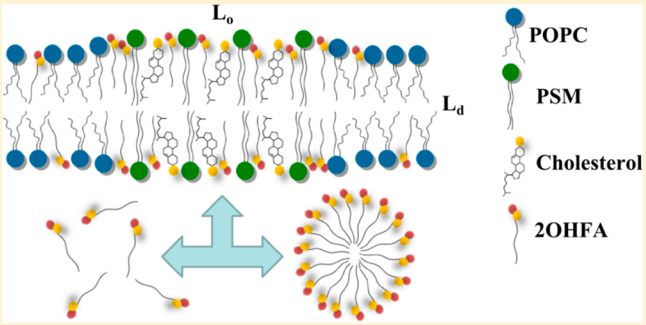
Supporting Information

ABSTRACT: Recent research regarding 2-hydroxylated fatty acids (2OHFAs) showed clear evidence of their benefits in the treatment of cancer, inflammation, and neurodegenerative disorders such as Alzheimer's disease. Monolayer compressibility isotherms and isothermal titration calorimetry of 2OHFA (C18−C22) in phosphatidylcholine/phosphatidylethanolamine/sphingomyelin/cholesterol (1:1:1:1 mole ratio), a mixture that mimics the composition of mammalian plasma membrane, were performed to assess the membrane binding capacity of 2OHFAs and their natural, nonhydroxylated counterparts. The results show that 2OHFAs are surface-active substances that bind membranes through exothermic, spontaneous processes. The main effects of 2OHFAs are a decrease in lipid order, with a looser packing of the acyl chains, and a decreased dipole potential, regardless of the 2OHFAs' relative affinity for the lipid bilayer. The strongest effects are usually observed for 2-hydroxyarachidonic (C20:4) acid, and the weakest one, for 2-hydroxydocosahexaenoic acid (C22:6). In addition, 2OHFAs cause increased hydration, except in gel-phase membranes, which can be explained by the 2OHFA preference for membrane defects. Concerning the membrane dipole potential, the magnitude of the reduction induced by 2OHFAs was particularly marked in the liquid-ordered (lo) phase (cholesterol/sphingomyelin-rich) membranes, those where order reduction was the smallest, suggesting a disruption of cholesterol−sphingolipid interactions that are responsible for the large dipole potential in those membranes. Moreover, 2OHFA effects were larger than for both lo and ld phases separately in model membranes with liquid disordered (ld)/lo coexistence when both phases were present in significant amounts, possibly because of the facilitating effect of ld/lo domain interfaces. The specific and marked changes induced by 2OHFAs in several membrane properties suggest that the initial interaction with the membrane and subsequent reorganization might constitute an important step in their mechanisms of action.



INTRODUCTION

The composition of the cell membrane in terms of fatty acids (FAs), mostly found as part of phospholipids and sphingolipids, can modulate important signaling transduction pathways and ultimately control vital physiological processes. For instance, the consumption of large amounts of olive oil, which is enriched in oleic acid (OA), reduces the incidence of cardiovascular diseases and cancer.[1,2] Docosahexaenoic acid (DHA) and eicosapentaenoid acid (EPA) have also been associated with the prevention of cardiovascular diseases and cancer[3] as well as with a strong neuroprotective effect by reducing amyloid β42 production.[4] In this sense, during the past few years, a number of 2-hydroxylated fatty acids (2OHFAs, Figure 1) have been designed as active drugs for lipid therapy related to cardiovascular issues,[5] cancer,[6,7] inflammation,[8] obesity,[9] and Alzheimer's disease.[10] 2-Hydroxyoleic acid (2OHOA), the most studied molecule of the group, may exert its antitumor effects through different mechanisms, including the induction of apoptosis or cellular differentiation and autophagy, where the sphingomyelin (SM) cell content seems to be involved.[6,7] 2-Hydroxyarachidonic acid (2OHARA) has a highly anti-inflammatory effect through interference with cicloxygenase expression and enzymatic activity,[8] and 2-hydroxydocosahexaenoic acid (2OHDHA) delivered promising effects in Alzheimer's disease.[10]

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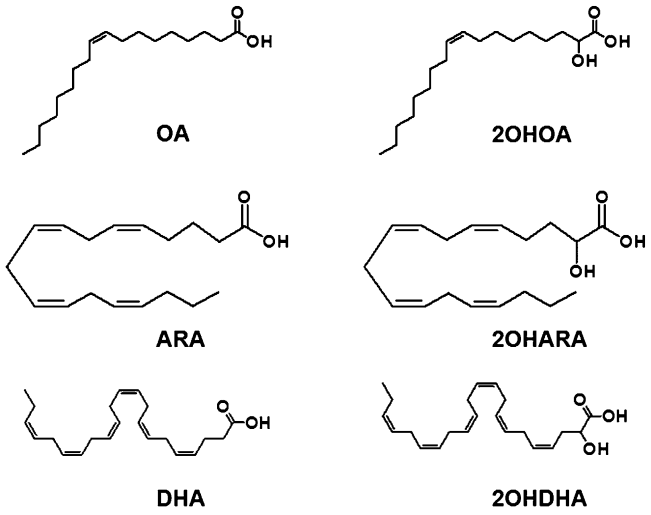

Figure 1. Chemical structures of the natural and synthetic fatty acids used in the study. OA, oleic acid (C18:1); ARA, arachidonic acid (C20:4); DHA, docosahexaenoic acid (C22:6); 2OHOA, 2-hydroxyoleic acid (C18:1); 2OHARA, 2-hydroxyarachidonic acid (C20:4); and 2OHDHA, 2-hydroxydocosahexaenoic acid (C22:6).

Free fatty acids (FFAs) are minor components of biological membranes accounting for ca. 1 to 2% of total lipids;[11] however, they are important components in certain membranes such as in the small intestine brush border where their levels can reach ca. 20% of total lipids.[12] Moreover, FFAs affect basic processes common to various cell types, which may answer the question of how these simple molecules can be involved in different, seemingly unrelated pathologies. The mechanism of action of FAs and 2OHFAs on cell signaling has been proposed to be linked to changes in membrane structure and function.[13] At high concentrations, the incorporation of FFAs into the lipid bilayer has a general solubilizing effect because of their ability to form micelles.[14] However, at smaller and possibly more physiologically relevant concentrations, FFAs may exert more subtle effects, slightly changing important biophysical parameters that have been shown to modulate the activity of membrane proteins,[15−17] such as lateral pressure,[18] membrane fluidity,[19] and microdomain reorganization.[13] These membrane biophysical properties can change because of either the incorporation of FFAs or alterations in the FA composition of acylated lipids. Nevertheless, the mechanism of action of these molecules is not fully understood.

A majority of studies concerning FAs and 2OHFAs examine the effect of the unsaturation/chain length/hydroxylation on lipid membranes through the use of phospholipids with different acyl chain compositions in model membranes or through metabolically induced changes in acyl chain compositions of cell membranes.[20−22] An increase in the number of double bonds in the *sn*-2 chain of monounsaturated phosphatidylcholine (PC) results in an increase in the lateral diffusion in one-component PC bilayers.[23] In the same study, a phase separation into liquid-disordered (ld) and liquid-ordered (lo) phases was observed in a ternary system of PC/egg SM/cholesterol (Chol) for more unsaturated PCs as a result of the increasing difficulty of incorporation of more unsaturated lipids into the highly ordered SM/Chol matrix. The ability of polyunsaturated fatty acids (PUFAs) to modify the membrane lipid raft structure and composition contributes to membrane protein function modulation and the activation of signaling cascades such as triggering the proapoptotic-ceramide-linked pathway.[24,25] In this context, treatment with 2OHOA leads to a marked increase in SM levels in cancer cells (in which basal SM levels are markedly low) among other lipid metabolism alterations, which strongly influences the membrane biophysical properties.[7]

Nevertheless, the effect of the direct addition of FAs or the increased concentration of FFAs (e.g., due to the activation of PLA_2[17,26,27]) can be different from FA incorporation into phospholipids. The effect of the direct insertion of FFA on the bilayer elastic properties has been described, and it was concluded that there were noticeable consequences of PUFAs addition, such as DHA, whereas saturated and monounsaturated FFAs were relatively inert.[16] The effects could be partially explained by negative curvature induction and plasma membrane lipid raft structure changes.[14] Also, the impact of different OHFAs on the dimyristoylphophatidylcholine (DMPC) model membrane phase transition was assessed through differential scanning calorimetry as a function of chain length and the position of the attached hydroxyl group. These studies showed a tendency of FAs with unsaturations and hydroxyl groups in the hydrophobic core of the membrane to reduce the lipid order as deduced by the decrease in the solid to liquid-crystalline phase transition temperature (T_m) values.[28] The presence of free 2OHOA in lipid bilayers was hypothesized to contribute to increased membrane hydration and fluid domain organization,[29] having a general fluidifying effect in lipid membranes.[13,30,31] Nonetheless, 2OHOA, 2OHARA, and 2OHDHA were shown to induce a higher hydration level and increased disorganization through increased acyl chain mobility and decreased lo domain size and proportion when added to membrane model systems.[13] Thus, it is important to know the effect of this addition and understand its contribution in comparison to 2OHFA incorporation into phospholipids and even sphingolipids.[32−34] This knowledge will allow a better understanding of the molecular mechanism of 2OHFAs with respect to their therapeutic action.

The present study examines the interactions of synthetic, unsaturated 2OHFAs molecules with potentially unique therapeutic properties and their naturally occurring counterparts with model membranes mimicking different lipid phases and phase coexistence situations in order to obtain a deeper understanding of their molecular mechanisms of action. Here, it is shown that the ability of 2OHFAs to reorganize the lipid bilayer microdomains toward a less organized state is not directly related to the degree of interaction of FAs and 2OHFAs with the membrane. The strongest changes are induced by 2OHARA, suggesting an important role of the initial interaction with the membrane and the domain reorganization in its mechanism of action. In single-phase bilayers, small changes in membrane order/compactness are observed, which are enhanced when ld/lo phases coexist. Moreover, marked changes occur in hydration properties, particularly in lo bilayers, pointing overall to a role in membrane microdomain organization with respect to the initial interaction of 2OHFA with the target cells.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine (POPC), liver bovine phosphatidylethanolamine (PE), 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylethanolamine (POPE), egg chicken SM, *N*-palmitoyl-SM (PSM), and Chol were purchased from Avanti Polar Lipids (Alabaster, AL). DMSO, OA, and arachidonic acid (ARA)

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were purchased from Sigma-Aldrich (St. Louis, MO). EPA and DHA were obtained from BASF Pharma (Callanish, U.K.). 2OHOA, 2OHARA, and 2OHDHA were kindly provided by Lipopharma Therapeutics, S.L. (Palma, Spain). Chloroform and methanol were purchased from Scharlab (Barcelona, Spain) and Fluka (St. Louis, MO, USA). Ethanol was also purchased from Fluka. Nuclepore and Anotop filters were purchased from Whatman (Kent, U.K.). The probe *trans*-parinaric acid (*t*-PnA) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA); 1,6-diphenyl-1,3,5-hexatriene (DPH) and di-4-ANEPPS were purchased from Invitrogen, Molecular Probes (Barcelona, Spain).

Preparation of Lipid Vesicles. For isothermal titration calorimetry experiments, large unilamellar vesicles (LUV) composed of POPC/PE/SM/Chol (1:1:1:1 mole ratio) were generated in a solution of 10 mM Hepes, 1 mM EDTA, and 100 mM NaCl at pH 7.4 using 100 nm filters according to previously described methods.[35] Quantitative analysis of the lipid composition of LUVs was performed by thin-layer chromatography in order to confirm that the final composition did not differ significantly from the initial lipid mixture.

For fluorescence measurements, appropriate volumes of POPC, POPE, PSM, and Chol stock solutions were mixed to mimic the lipid composition of a mammalian plasma membrane (equimolar mixture), U-118 glioma cell membrane,[7] and the main types of lipid phases (gel, lo, and ld)[36] (Table 1). Lipids were suspended in a solution of 10 mM Hepes, 150 mM NaCl, and 0.1 mM EDTA at pH 7.4 to obtain multilamelar vesicle (MLV) suspensions at a final lipid concentration of 2 or 3 mM. To obtain LUV, MLV suspensions were extruded using an Avanti miniextruder and nuclepore polycarbonate filters (100 nm pore diameter). Aliquots from the total LUV suspension with a final lipid concentration of 0.5 mM (which ensures the efficient incorporation of 2OHOA into the lipid bilayer[30]) were labeled with either DPH, *t*-PnA, or di-4-ANEPPS (1:500 probe/lipid ratio) added from stock ethanol solutions and incubated for 1 h at 50 °C.[37,38] For a direct comparison with the ITC results, the experiments with the equimolar mixtures were also performed for 0.1 mM lipid to preserve the same lipid/FA proportions and using the same hydration buffer as in the ITC experiments, yielding essentially the same results (not shown). Ethanol was added in a concentration that did not disturb the lipid bilayer properties.[39] The suspension was slowly brought to room temperature and allowed to equilibrate, and then 20 mol % 2OHFA was added and incubated for at least 1 h before fluorescence measurement.

Table 1. Mole Fraction of the Lipids Used to Mimic the Lipid Composition of the Mammalian Plasma Membrane, U-118 Cells Plasma Membrane,[7] and the Main Types of Lipid Phases[36]

	mole %				
	mammalian plasma membrane model	U-118 glioma cell membrane	ld	lo	gel
POPC	25.0	36.2	71.8	25.2	
POPE	25.0	23.6			
PSM	25.0	6.8	23.1	35.5	100
cholesterol	25.0	33.4	5.1	39.3	

Surface Pressure Measurements. Changes in surface pressure (Π) were measured on a Delta Pi-4 Kibron instrument (Helsinki, Finland) equipped with four-channel Langmuir tensiometers. Natural and synthetic FAs were dissolved in DMSO to a final concentration of 1 mM, and they were injected into the subphase of a trough filled with a solution of 10 mM Hepes, 1 mM EDTA, and 100 mM NaCl at pH 7.4 under continuous stirring. After the addition of FA and 2OHFA, the system was left to equilibrate for at least 10 min and the Π value was recorded. The analysis of FA or 2OHFA insertion into lipid membranes was performed using POPC/SM/PE/Ch (1:1:1:1 mole ratio) as a membrane substrate. In brief, the lipid mixture dissolved in chloroform/methanol (2:1 vol/vol) was added dropwise to the buffer surface to reach the initial surface pressure (Π_i). Because the Π_i value cannot be controlled accurately enough, values of 0.3−0.5 mN/m under or above the desired values were considered acceptable. The organic solvent was left to evaporate, and the FA or 2OHFA dissolved in DMSO was injected into the subphase until the Π value did not change. Increases in Π were plotted against the Π_i values, and data were fitted to a straight line as described elsewhere.[40] The small changes in Π_i induced by DMSO alone were subtracted from the raw data.

Isothermal Titration Calorimetry. ITC measurements were performed at 298 K in a VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA). A 10 mM LUV suspension (2 μL) was injected into an ITC cell containing 30 μM FA or 2OHFA. The thermodynamic parameters of 30 injections were recorded and analyzed using the MicroCal Origin software. The apparent binding constant K_a ($K_d = 1/K_a$) and enthalpy $\Delta H°$ were obtained from the fitting curve analysis of isotherms. The model for the calculation of K_d shown in the present study has been extensively used elsewhere,[41,42] and although it represents a global measurement of the free energy of interaction, which could in principle be decomposed into several partial steps/contributions, it allows the study of similarities and differences between the studied natural and synthetic FAs. The Gibbs free energy ($\Delta G°$) and entropy ($\Delta S°$) of binding are determined from the expression

$$\Delta G° = \Delta H° - T\Delta S° = -RT \ln K_a \quad (1)$$

Fluorescence Measurements and Data Analysis. Fluorescence measurements were made at 24 °C using a Horiba Jobin Yvon Spex Fluorolog 3-22/Tau 3 spectrofluorometer (Kyoto, Japan).

For steady-state fluorescence anisotropy measurements, the samples were excited at 303, 358, and 465 nm and emission was collected at 404, 430, and 610 nm for *t*-PNA, DPH, and di-4-ANEPPS labeling, respectively. For wavelength scans, the excitation and emission wavelengths are indicated in the Results section. The membrane dipole potential was measured through the excitation ratio of intensities at 420 nm/520 nm of di-4-ANEPPS with emission at 610 nm.[37,43] The steady-state anisotropy $\langle r \rangle$ was calculated according to eq 2[44]

$$\langle r \rangle = \frac{\left(I_{VV} - \frac{I_{HV}}{I_{HH}} \times I_{VH}\right)}{\left(I_{VV} + 2\frac{I_{HV}}{I_{HH}} \times I_{VH}\right)} \quad (2)$$

in which I_{XY} represents the emission intensity reading with vertical (V) or horizontal (H) orientations of the excitation (X) and emission (Y) polarizers. An adequate blank was subtracted from each intensity reading, and each set of four intensity components for each sample was measured seven times. Three nanometer bandwidth was used for DPH, and 4 nm bandwidth was used for *t*-PNA and di-4-ANEPPS.

For time-resolved measurements by the single-photon-counting technique (SPT), nanoLED N-310, N-370, and N-460 were used for the excitation of *t*-PNA, DPH, and di-4-ANEPPS, and emission wavelengths were 404, 430, and 610 nm, respectively. The resolution of the detection system was 50 ps. The number of counts in the peak channel was 10 000−20 000 for each sample. The time scale used for the analysis varied from 0.05552 ns/channel (DPH, di-4-ANEPPS, and *t*-PnA in ld membranes) to 0.11104 ns/channel (*t*-PnA in ld/lo and lo membranes) to 0.22312 ns/channel (*t*-PnA in gel membranes). The bandwidth was adjusted from the maximum value allowed by the instrumental setup (15 nm) to ensure an SPT regime (15 nm for di-4-ANEPPS; 6−15 nm for both *t*-PnA and DPH). Data analysis was performed through a nonlinear least squares iterative reconvolution method based on the Marquardt algorithm using the Time-Resolved Fluorescence Anisotropy Data Processor 1.4 program to obtain the fitting parameters, as previously described.[32]

For a fluorescence intensity decay described by a sum of exponentials,

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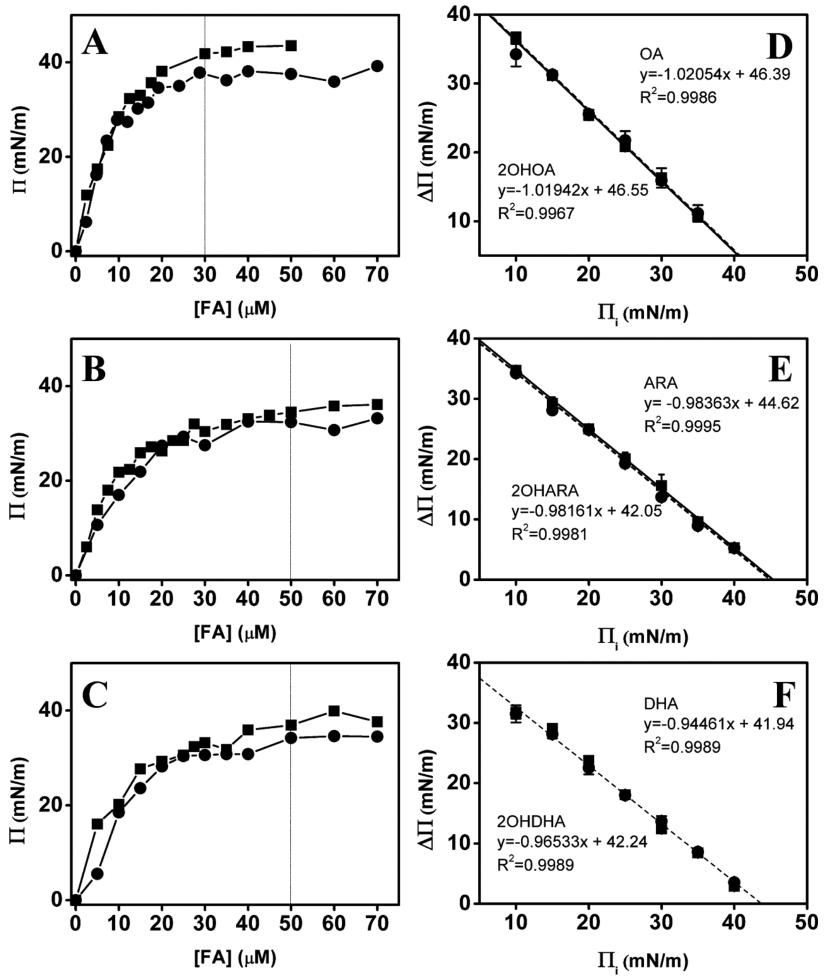

Figure 2. Surface pressure measurements of FA or 2OHFA in model membranes. Tensioactive properties of (A) OA or 2OHOA, (B) ARA or 2OHARA, and (C) DHA or 2OHDHA. The increase in Π in the buffer−air interphase was recorded at increasing concentrations of natural and hydroxylated FAs injected into the subphase. (D−F) Insertion of 30 μM FA or 2OHFAs into phospholipid monolayers composed of POPC/SM/PE/Chol (1:1:1:1 mole ratio). Changes in Π induced by (D) OA or 2OHOA, (E) ARA or 2OHARA, and (F) DHA or 2OHDHA were plotted against the Π_i values of the preformed phospholipid monolayer. Naturally occurring fatty acids (OA, ARA, and DHA) are shown as filled squares whereas synthetic 2-hydroxylated fatty acids (2OHOA, 2OHARA, and 2OHDHA) are represented by filled circles. Data represent mean values ± SD of three independent experiments.

$$i(t) = \sum_i a_i \exp\left(\frac{-t}{\tau_i}\right) \quad (3)$$

α_i and τ_i are the normalized amplitude and lifetime of component i, respectively. The intensity-weighted mean fluorescence lifetime is given by

$$\langle\tau\rangle = \frac{\sum_i a_i \tau_i^2}{\sum_i a_i \tau_i} \quad (4)$$

Two to four exponentials were required to describe each of the fluorescence intensity decays, depending on the lipid mixture composition and the probe under analysis. The background (obtained with the corresponding blank sample) was subtracted from the decay.

All data are expressed as the mean ± standard deviation of at least three independent samples.

FA Oxidation Assay. Fatty acid oxidation was measured by light absorption at 245 nm.[45] One mg/mL OA or 2OHOA and 0.05 mg/mL ARA, 2OHARA, DHA, or 2OHDHA were incubated at room temperature in 2 mL of a solution of 10 mM Hepes, 1 mM EDTA, and 100 mM NaCl at pH 7. The incubation time was 60 min, which exceeds the experimental conditions of the surface pressure measurements. FAs were extracted by the addition of chloroform/methanol (2:1 vol/vol), followed by evaporation of the lower organic phase. The lipid film was resuspended in ethanol, and absorption at 245 nm was recorded in a Varian Cary 300 Bio spectrophotometer (Palo Alto, CA). For negative oxidation controls, lipids were incubated in the presence of 50 μM butylated hydroxytoluene, and they were immediately extracted as above. As a control for the oxidative state, FA or 2OHFA solutions were incubated at room temperature for 24 h in the absence of EDTA and in the presence of 12 μM $CuSO_4$.

RESULTS AND DISCUSSION

Incorporation of Fatty Acids in Model Membranes. FAs are amphiphilic molecules with a propensity to insert into lipid bilayers. Previous studies have shown, by means of molecular dynamics models, that the incubation of both OA and 2OHOA with PC bilayers provokes the aggregation of FAs in the first steps of the simulations, followed by their spontaneous insertion into the bilayer.[31] To check the tensioactive properties and the tendency of natural and synthetic FAs to interact with lipid membrane, surface pressure studies were performed at the air−water interface. Figure 2A−C shows mean curves of equilibrium values of Π after the addition of different FA or 2OHFA concentrations to the subphase of a tensiometer. 2OHFA and FA caused a dose-

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Table 2. Thermodynamic Parameters for FA−and 2OHFA−Membrane Interaction Obtained from Isothermal Titration Calorimetry Using LUVs Composed of POPC/SM/PE/Chol (1:1:1:1 Mole Ratio)

fatty acid	K_d (μM)[a]	$\Delta H°$(kcal/mol)[a]	$-T\Delta S°$(kcal/mol)[a]	$\Delta G°$ (kcal/mol)[a]
OA	3.65 ± 0.18	−3.09 ± 0.01	−4.32 ± 0.03	−7.41 ± 0.04
2OHOA	28.92 ± 3.02[c]	−1.46 ± 0.40[c]	−4.79 ± 0.41	−6.22 ± 0.01[d]
ARA	49.38 ± 11.50	−1.62 ± 0.50	−4.89 ± 0.36	−5.7 ± 0.28
2OHARA	27.86 ± 7.50	−3.43 ± 0.65	−3.85 ± 0.25	−7.28 ± 0.90
DHA	39.24 ± 5.80	−5.00 ± 0.92	−1.89 ± 0.40	−6.89 ± 1.30
2OHDHA	4.37 ± 2.86[c]	−3.99 ± 0.19	−3.48 ± 0.27[b]	−7.47 ± 0.46

[a]Mean value ± SD. Statistics comparing FA with 2OHFA. [b]$p < 0.05$. [c]$p < 0.01$. [d]$p < 0.001$.

dependent increase in Π with a maximum value of ca. 40 mN/m when using 30 μM OA or 2OHOA (Figure 2A). However, when polyunsaturated ARA, 2OHARA, DHA, and 2OHDHA (Figure 2B,C) molecules were used, a plateau of ∼35−40 mN/m was reached at a higher FA concentration (ca. 50 μM). This concentration value probably represents the point at which FA and 2OHFA micelles start to form so that a regime of monomers in solution in equilibrium with monomers at the interface is being replaced by a more complex equilibrium among monomers (interface), monomers (solution), and micelles (solution). These results show that these FA and 2OHFA molecules are surface-active lipids with a critical micellar concentration in the 30−50 μM range, a concentration used for the study of incorporation of 2OHFA or FA into preformed phospholipid model membranes. Critical micellar concentration values are notoriously dependent on the method used. In this work, we have used the surface pressure method that is widely accepted and has the advantage of not using external probes.[46−48]

The insertion of 2OHFA and FA into phospholipid monolayers was tested using the Langmuir balance, with preformed POPC/PE/SM/Chol (1:1:1:1 mole ratio) monolayers at the air−buffer interface and FA or 2OHFA injected into the subphase. Increases in lateral pressure due to FA or 2OHFA insertion depended strongly on the Π_i value. The increments of surface pressure ($\Delta\Pi$) upon 2OHFA or FA insertion into the preformed phospholipid monolayer were plotted as a function of Π_i (Figure 2D−F). A straight line was fitted to the data, and extrapolation to $\Delta\Pi = 0$ yielded the maximum monolayer surface pressure after the insertion of the studied molecule into the monolayer. 2OHFA or FA may be inserted into monolayers to resemble cell membrane compositions of up to ca. 40−46 mN/m for either the polyunsaturated FA or 2OHFAs. This is important because cell membranes are considered to support a lateral pressure of ∼30 mN/m,[49] albeit with large fluctuations around this average value. Thus, the data in Figure 2D−F indicate that these molecules can insert easily into cell membranes, in agreement with their well-known effects on the cellular level.[7] No noticeable differences were registered between 2OHFA and their natural nonhydroxylated FA analogues. Moreover, the maximum pressure supported by POPC/PE/SM/Cho (1:1:1:1 mole ratio) monolayers is ∼44−46 mN/m (data not shown). According to Figure 2D−F, the collapse pressure of the monolayer upon insertion of FA or 2OHFA reaches a similar Π value, indicating that neither FA nor 2OHFA affects the monolayer stability to collapse.

Lipid oxidation was determined spectrophotometrically in order to discard any influence of FA and 2OHFA degradation during the course of the experiment (Figure S1 in Supporting Information). Neither FA nor 2OHFA was oxidized under the conditions of the experiments, as described in Materials and Methods. Concerning the effect of DMSO solvent on lipid membranes, it has already been described that under our conditions DMSO does not alter the structure of giant unilamellar vesicles (GUVs) or LUVs.[13] In addition, the small contribution of DMSO to the increase in Π was subtracted individually from each data point. Thus, the addition of 80 μL of DMSO, which corresponds to the maximum volume used in the study of surface-active properties, induced an increase of 1.8 mN/m whereas the same volume of vehicle containing 70 μM FA or 2OHFA yielded a Π value increment of 35−40 mN/m.

Membrane Binding Thermodynamic Parameters of Fatty Acids. ITC was used to study the capacity of different natural and synthetic FAs to bind to model membranes mimicking the plasma membrane composition (POPC/PE/SM/Chol 1:1:1:1 mole ratio). Although the interaction of FA and 2OHFA with the lipid bilayers is much more complex than the binding of a drug to a specific site in a protein, a simple model considering one binding constant gave an excellent description of the ITC data and, as described in Materials and Methods, was used to compare the relative affinity of the molecules for the membrane and to assess the total free energy of the membrane-FA interaction, which should not be regarded just as the free energy of binding. The use of a simple model is also justified by the observation that the membrane/water partition constant of several FA, including OA and DHA, is independent or very weakly dependent on membrane composition and is independent of FA concentration at least in the range of micromolar to tenths of micromolar.[16,50] Table 2 shows a similar relative affinity of DHA and OA for the lipid bilayers as in previous work,[16] even though it was obtained via a different method, further supporting the use of the model here described.

In this context, OA (3.65 ± 0.18 μM) and 2OHDHA (4.37 ± 2.86 μM) showed a higher binding affinity for the lipid bilayer than did other FA or 2OHFA molecules (Table 2); ARA had the lowest affinity value (49.38 ± 11.5 μM) for the studied membrane model whereas 2OHOA had an intermediate affinity (K_d = 28.92 ± 3.02 μM) (Figure 3 and Table 2). Moreover, all of the binding processes were exothermic ($\Delta H° < 0$), increased the disorder of the system ($\Delta S° > 0$) and were therefore spontaneous ($\Delta G° < 0$). No marked differences in $\Delta G°$ values were observed for the six natural and synthetic FAs studied. The spontaneity of the process is in agreement with the Langmuir balance results here presented, where it was found that all of the studied natural and synthetic fatty acid molecules had a tendency to insert into the lipid membrane.

Membrane Reorganization by 2-Hydroxylated Fatty Acids. To study the effect of 2OHFAs on membrane organization, vesicles mimicking the lipid composition of the mammalian plasma membrane (POPC/POPE/SM/Chol,

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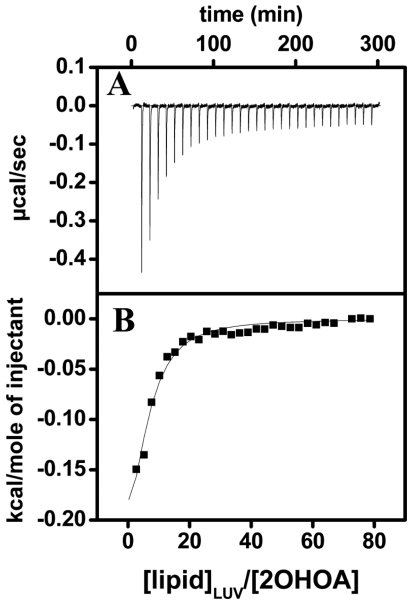

Figure 3. Isothermal titration calorimetry assay of the binding of 2OHOA to LUV composed of POPC/PE/SM/Chol (1:1:1:1 mole ratio). (A) Representative titration experiment of LUV (10 mM) and 2OHOA (30 μM) and (B) integrated data (■) and one-site fitting curve of the binding isotherm from panel A.

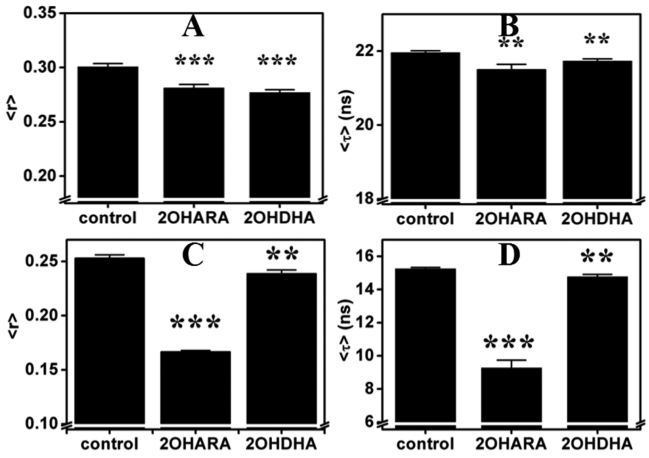

Figure 4. Fluorescence spectroscopy analysis of membrane order and compactness in POPC/POPE/SM/Chol liposomes mimicking (A, B) the mammalian plasma membrane and (C, D) the U-118 cell membrane lipid composition (molar proportions in Table 1) incubated with polyunsaturated 2-hydroxylated fatty acids. (A, C) DPH steady-state fluorescence anisotropy (3 nm bandwidth). (B, D) *t*-PnA mean fluorescence lifetime. Statistics comparing the control with the 2OHFA-treated sample: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

1:1:1:1 mole ratio) were analyzed in the presence or absence of each 2OHFA by fluorescence spectroscopy (fluorescence anisotropy and lifetime and excitation/emission spectra). Three different probes that are particularly sensitive to certain types of phases and/or membrane biophysical properties were used. More specifically, the global membrane order was assessed by measuring the DPH fluorescence anisotropy because this probe has no preference for lipid domains and is aligned with the phospholipid acyl chain palisade (ref 51 and references therein). *t*-PnA is especially useful in detecting the presence of a gel phase because its fluorescence lifetime is highly dependent on the acyl chain packing, presenting a long-lifetime component in ordered domains.[32,52] The probe di-4-ANEPPS is very sensitive to water penetration and H-bonding patterns because of the very large change in its dipole moment upon excitation in the direction of the membrane normal.[53] Because of its shallow location in the lipid bilayer as compared to that of the other two probes, it detects the alterations in biophysical properties mainly in the lipid headgroup/water interface region.[43] It can also be used to measure membrane dipole potential alterations. Moreover, these properties are strongly affected by "raft-forming" sterols,[54] and thus this probe is sensitive to Chol-enriched domains.[37]

The general effect of 2OHARA and 2OHDHA in model membranes mimicking the composition of the mammalian plasma membrane (POPC/POPE/SM/Chol, 1:1:1:1 mole ratio) is the reduction in lipid order and packing in the membrane (Figure 4A,B), as had already been shown for 2OHOA.[13,30] This effect is manifested by a decrease in both DPH steady-state fluorescence anisotropy and the *t*-PnA mean fluorescence lifetime. Furthermore, this effect seems to be independent of the capacity of 2OHFA to bind to the lipid bilayer. For instance, 2OHDHA, the fatty acid derivative that binds to the assayed membranes with the highest affinity (Table 2), induces less disorganization in liposomes than do other 2OHFAs, according to recent NBD self-quenching data[13] and the mean fluorescence lifetime of *t*-PnA (Figure 4B).

LUVs with lipid compositions corresponding to three different lipid phases at room temperature (gel, ld, and lo) were also analyzed in order to study separately the effect of 2OHFA in bilayers mimicking different types of membrane domains (Figure 5). The lipid compositions used (Table 1) for ld and lo phases correspond to the ends of the same tie line in the phase diagram in order to ensure their possible coexistence in a phase-separated mixture (i.e., a raft-containing membrane model[36]). As expected, in the control samples the steady-state fluorescence anisotropy decreases in the order gel > lo > ld, with values similar to those previously reported for the gel phase (>0.3), lo (0.2−0.3), and ld (<0.2)[36,38] (Figure 5A). Regarding the *t*-PnA mean fluorescence lifetime, control values were as expected above 30 ns for the gel phase, at ∼20 ns for lo, and at ∼10 ns for ld (Figure 5B). With the addition of any 2OHFA, both DPH steady-state fluorescence anisotropy and *t*-PnA mean fluorescence lifetime values diminished, pointing toward membrane fluidification with a looser packing of the acyl chains (Figure 5A,B). Furthermore, the di-4-ANEPPS steady-state fluorescence anisotropy also decreased, meaning that the probe has faster and/or less restrained rotational dynamics, indicating that 2-OHFA induces looser packing in the lipid headgroup region of the bilayer (Figure 5C). In fact, the trend observed for di-4-ANEPPS fluorescence anisotropy (Figure 5C) is parallel to those of DPH (Figure 5A) and *t*-PnA (Figure 5B). The results show a similar tendency toward membrane order changes with the three probes tested (DPH, Figure 5A; *t*-PnA, Figure 5B; and di-4-ANEPPS, Figure 5C) independently of the lipid phase, with a greater effect observed for 2OHARA than for either 2OHDHA or 2OHOA. Interestingly, lo was the least-affected type of membrane phase by any of the different 2OHFA derivatives.

This disordering effect is also accompanied by changes in the hydration state, solvent relaxation dynamics, and dipole potential. This is evidenced by both shorter fluorescence lifetimes of di-4-ANEPPS in ld and lo membranes (not shown)

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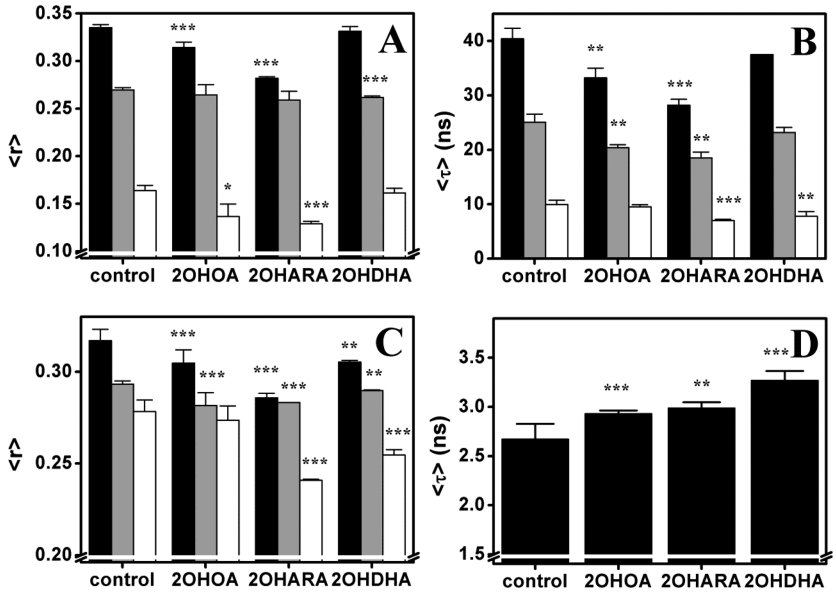

Figure 5. Fluorescence spectroscopy analysis of membrane order and compactness in lipid vesicles with compositions corresponding to a single phase, gel (filled bars), lo (gray bars), or ld (empty bars), as described in Table 1, incubated with 2-hydroxylated fatty acids. (A) DPH steady-state fluorescence anisotropy (3 nm bandwidth). (B) *t*-PnA mean fluorescence lifetime. (C) Di-4-ANEPPS steady-state fluorescence anisotropy (4 nm bandwidth). (D) Di-4-ANEPSS mean fluorescence lifetime in liposomes of SM in the gel phase incubated with 2-hydroxylated fatty acids. Statistics comparing the control with the 2OHFA-treated sample: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

and spectral red shifts in the emission of di-4-ANEPPS dye for the three phases that will be described below (Table 3), which

Table 3. Di-4-ANEPPS (1/500 Probe/Lipid Ratio) Excitation and Emission Spectral Maxima Wavelengths in Liposomes with Compositions Corresponding to a Single Gel, ld, or lo Phase with Compositions Given in Table 1 and Spectral Shifts Induced by the 2-Hydroxylated Fatty Acids

	gel		lo		ld	
	λ_{ex}(nm)	λ_{em}(nm)	λ_{ex}(nm)	λ_{em}(nm)	λ_{ex}(nm)	λ_{em}(nm)
control	475	622	462	582	468	618
2OHOA	477	624	472	597	472	618
2OHARA	484	617	481	622	476	621
2OHDHA	481	618	472	597	467	618

are due to a stronger contact of the probe with polar water molecules. Regarding the fluorescence lifetimes, exceptional behavior was observed for gel-phase LUVs, which are formed by a single lipid, SM. In this type of phase, it is well known that line defects, which are analogous to crystal grain boundaries, exist and are able to accumulate "impurities" as a result of their increased hydration.[55,56] As can be observed in Figure 5D, the di-4-ANEPPS excited-state mean fluorescence lifetime becomes longer in the presence of 2OHFA, with a more pronounced effect for 2OHDHA. This exceptional behavior can be explained by the fatty acids' preference for the line defects. An occupation of those defects would reduce the hydration, thereby increasing the di-4-ANEPPS fluorescence lifetime. This is consistent with the order in which the effect is observed: 2OHOA has an acyl chain that is more similar to the egg SM acyl chain and sphingoid base length[57] and therefore should be the one that can be better accommodated in nondefect regions. On the contrary, 2OHDHA, having the longer acyl chain and the highest number of unsaturations, should have the opposite behavior, showing an extensive preference for the defects and thus the larger effect in reducing the hydration of the gel phase. In fact, for DHA, several conformations are predicted, and for those with the most extreme bending, it is not expected that they are easily accommodated between the phospholipid acyl chains.[14]

In the three types of lamellar lipid phases relevant to animal cell membranes and in the situation of ld/lo coexistence (quaternary mixture), the membrane alterations induced by the fatty acids corresponded to a decrease in the order/compactness and increased hydration of the membrane (except for the single gel phase situation). The effects were always more marked for 2OHARA than for 2OHDHA and 2OHOA. Therefore, our results suggest that the FAs have the ability to interact with the three types of lipid domains when having their first contact with the target cell membrane. Moreover, this general disordering effect is expected to contribute to remixing of the lipids between ordered and disordered domains. The preference shown for the line defects of the gel phase suggests that, as already known for a large number of small amphiphilic molecules, they may somehow prefer to incorporate into membrane defects that are more likely to be present at the interfaces between ordered and disordered domains, reducing the line tension between them and leading to smaller or less-well-defined areas. This last feature was indeed recently observed on GUV.[13]

Interaction with Liquid Ordered Domains and ld/lo Phase Coexistence Mixtures. In both quaternary mixtures used in this work, there is a coexistence of ld and lo phases, as observed by confocal microscopy in GUVs[13] and predicted by mapping their composition on an experimentally determined phase diagram.[7] In this case, probes DPH and *t*-PnA were used because they have ld/lo partition coefficients close to 1 in a variety of binary and ternary Chol-containing mixtures.[58,59] Therefore, the changes undergone in DPH anisotropy and in the *t*-PnA mean fluorescence lifetime could be interpreted straightforwardly as alterations in phase properties and

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proportion and are not affected by possible changes in the probe partition behavior. Interestingly, the fluidizing effect of 2OHFA is very weak in the lo phase (Figure 5A–C, gray bars), which could explain the low amplitude of the effect on DPH steady-state anisotropy in the equimolar quaternary mixture (Figure 4A). The control value in this mixture is near the upper limit for an lo phase, implying a high proportion of the lo phase in the phase coexisting mixture as also observed in GUVs.[13] However, in liposomes mimicking the U-118 glioma cell membrane (Table 1), the fluidizing effect of 2OHFA, particularly that of 2OHARA, was much more pronounced (Figure 4C). For this composition, the values of the control were lower than for the equimolar system regarding both the DPH anisotropy (Figure 4C) and the *t*-PnA fluorescence lifetime (Figure 4D), which indicates a smaller proportion of the lo phase than for the equimolar mixture, in agreement with the prediction carried out by mapping such a composition in the phase diagram.[7] This lower proportion of the lo phase justifies, to a large extent, the more marked effects of 2OHOA when compared to those in the equimolar mixture. Moreover, the significantly smaller amount of SM (Table 1) should correspond to a higher preponderance of nanometer- rather than micrometer-sized domains,[38] which would correspond to a larger fraction of lipids in the ld/lo domain interfaces. Finally, the effect on U-118 model membrane systems was larger than for both lo and ld phases separately (Figure 5), suggesting that ld/lo phase coexistence plays a role in the initial interaction of 2OHFA with the membrane. For example, upon addition of 2OHARA the anisotropy of DPH is decreased by ∼35% in U-118 cell membrane models, whereas the decrease was only ∼20% in ld membranes and not significant in lo. When the *t*-PnA mean fluorescence lifetime is used, similar conclusions can be reached. Thus, the changes observed in the single-phase systems cannot account for the extent of the alterations observed for ld/lo coexistence, showing that the presence of two phases enhances the effects of 2OHARA. Note that the compositions of the pure ld and pure lo phases were chosen to be extremes of the same tie line. The extent of the effect observed on bilayer order and compactness for the U-118 model strongly suggests that the fraction of lo domains is significantly diminished; concomitantly, the ld phase becomes the main one. However, this does not happen for the pure lo phase system or for the ld/lo system with a greater lo phase fraction (equimolar mixture), where in principle the extent of such an effect could be larger because there is more lo that can be turned into ld. Thus, the lo domains in the U-118 membrane model are much more susceptible to the action of 2OHARA, and there is clearly no linear relation between the effects observed and the fraction of the phases. This can in principle indicate the important role of domain structure because as described above not only is the fraction of lo lower but also the domain size is smaller in the U-118 model, which corresponds to a higher relative amount of domain boundary lipid when compared to the equimolar mixture. This interfacial lipid could facilitate the access of more 2OHFA molecules to the more rigid lo domains and exert a stronger effect on them.

Di-4-ANEPPS is an environmentally sensitive probe that is particularly suited to investigating the effects of Chol, and this probe was used to access the possible role of lipid rafts in the 2OHFA interaction with model membranes in each of the three main lamellar phases (ld, lo, and gel). The excitation and emission spectra of di-4-ANEPPS (Table 3 and Figure S2) were analyzed, and it was observed that the effects on hydration patterns at the lipid–water interface induced by 2OHFA were much larger for the lo phase than for the others. This contrasts with the effects observed for DPH steady-state fluorescence anisotropy and *t*-PnA fluorescence mean lifetime data, suggesting a possible role of Chol (or lipid rafts). Chol induces a strong blue shift of di-4-ANEPPS fluorescence emission as a result of the reduced hydration and change in solvent relaxation dynamics, accompanied by a reorientation of the phospholipid headgroup or surrounding water molecules and therefore reorganization of the H-bonding patterns in the presence of Chol.[37] However, upon incubation with 2OHFA the extent of the shift is significantly reduced. In the case of 2OHARA, this effect is so strong that the wavelength of maximum emission becomes the same as that observed for the ld and gel membranes (i.e., in those membranes that contain as few as 5 mol % cholesterol or do not contain sterol at all, respectively (Table 3)). Thus, the consequence of 2OHFA incorporation into the membranes is not restricted to a disordering of the acyl chains, but it also significantly affects the hydration and solvent relaxation dynamics.

The excitation spectra of di-4-ANEPPS also contain information on the membrane dipole potential, a feature that is significantly enhanced by certain sterols, namely Chol. This biophysical property can be expressed as a ratio of excitation intensity at two wavelengths, which was calculated and is shown in Figure 6. The ratio of intensities is a suitable way of

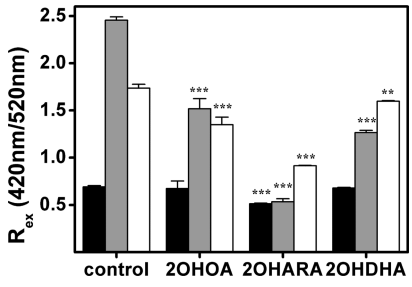

Figure 6. Membrane dipole potential measured through the excitation intensity ratio at 420 nm/520 nm for di-4-ANEPPS in single-phase liposomes (gel (filled bars), lo (gray bars), and ld (empty bars)) with compositions given in Table 1 and the effect of the indicated 2OHFA. Statistics comparing the control with the 2OHFA-treated sample: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

representing dipole potential alterations because there is a close to linear relation between them and also because the use of a ratio amplifies the range of possible values when compared to single-wavelength measurements and is therefore a much more sensitive parameter. Figure 6 shows that the membrane dipole potential was decreased upon FA insertion into the bilayer. The effect was stronger for 2OHARA than for 2OHDHA and 2OHOA, and its magnitude increased in the order gel < ld < lo. In the case of 2OHARA and the lo phase, the dipole potential decreased to values measured in the liposomes devoid of Chol (i.e., in the gel phase), suggesting that it is able to override the sterol-induced dipole potential. Again, it is shown that in the lo phase the general disordering effect is small but significant changes in other biophysical properties occur upon insertion of the 2OHFA. The alterations observed in the interfacial region hydration patterns and dipole potential indicate that the lo phase becomes much more similar to the other phases in terms of these properties. In addition, it justifies once more the

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intermixing of ld and lo structures to produce smaller microdomains, as previously observed in GUVs.[13]

Membrane Incorporation versus Lipid Bilayer Order Effect of 2OHFA. The overall membrane effects of 2OHFAs on lipid order are not related to their incorporation ability, as evidenced by the fact that 2OHDHA, the 2-hydroxylated molecule with the highest membrane affinity (Table 2), had the lowest effect on membrane domain remodeling. Moreover, 2OHARA has a greater effect on the membrane lipid order than 2OHOA, but both share a K_d of the same order of magnitude. A smaller disordering effect of 2OHOA is consistent with the changes induced in membrane elastic properties of their nonhydroxylated counterparts.[16] On the basis of previous studies of the OHFA effect on DMPC model membranes, the general disordering effect of 2OHFA in the present work can be primarily attributed to the unsaturation of the acyl chain, as evidenced by the fact that the 2OH group was shown to stabilize the gel phase, increasing the T_m of DMPC.[28] The smaller disorganizing effect of 2OHDHA can be explained by its longer acyl chain in comparison to that of the other 2OHFA, which opposes the disordering effect of the greater number of double bonds. In addition and as mentioned above, highly bended conformations of 2OHDHA (by analogy to DHA[14]) may adsorb more superficially to the lipid bilayer, and only a fraction will penetrate the acyl chain region. However, 2OHARA more easily penetrates the lipid bilayer, and its polyunsaturation will have a strong disordering effect.

When analyzing the consequences of lipid composition changes in U-118 cells treated with 2OHOA, it was shown that 2OHOA increases the order of the lo domains in membrane models and reconstituted liposomes as a result of the accumulation of SM in 2OHOA-treated cells.[7] When the direct effect of 2OHOA insertion was evaluated without taking into account the changes in lipid composition due to lipid metabolism alterations, a small disordering effect was observed, and the values retrieved for both DPH fluorescence anisotropy and *t*-PnA average fluorescence lifetime[30] fit the general trend presented here for the three 2OHFAs. We demonstrate here that the disorganization effect due to 2OHFA also occurs in quaternary lipid mixtures used as a model of a mammalian plasma membrane and in LUVs with compositions corresponding to different lipid phases, showing that this is a general effect of the unsaturated 2OHFA.

The decrease in the order/compactness of the phospholipid acyl chains due to 2OHFA incorporation, more obvious in gel and ld phase membranes, is also accompanied by a decrease in the membrane dipole potential and increased hydration of the membrane, with these latter effects being more noticeable for the membranes in the lo phase, followed by ld (i.e., for Chol-containing membranes). Previous studies of X-ray scattering patterns and differential scanning calorimetry heating thermograms have also pointed to increased hydration and membrane fluidity due to 2OHOA incorporation in phase coexisting mixtures (DOPC/SM/Chol 35:35:30 mole ratio).[29] Our results also show that with the exception of the gel-phase hydration that decreases, in all cases there is increased water penetration and reorientation of water molecules at the membrane/water interface, as evidenced by the emission red shift provoked by the 2OHFA and alterations of the membrane dipole potential (Table 3).

Role of Lipid Lateral Organization on Membrane–2OHFA Interactions. Our results also suggest an important role for lipid domains because the 2OHFA effect in the ld/lo phase coexistence system is overall larger than for lo and ld phases separately. Indeed, the comparison of the data in Figures 4C,D and 5 indicates that the phase coexistence is enhancing the effect of the 2OHFA. Interestingly, in case of the gel phase the effects are more marked and more similar to the quaternary mixtures, which may indicate that gel grain boundaries in one case and ld/lo domain boundaries in the other case are important for the interaction. This suggests that phase coexistence has an important role in the initial interaction of 2OHFA with the membrane, especially for 2OHARA. The difference between the amplitude of the membrane disordering effect in the equimolar and U-118 phase coexisting mixtures can be explained by a higher lo proportion in the former. The fact that 2OHFA exerts changes of different magnitudes in these two model systems suggests that the lipid composition and membrane microdomain organization of a given cell type might be relevant to the mechanism of action of the FA in that particular cell type. This would partially explain the observation of different anticancer mechanisms (cell cycle arrest, differentiation, and apoptosis) for 2OHOA when using different cell lines.[6,7]

The effect of the direct incorporation of 2OHFA into the global order of the membrane (DPH steady state fluorescence anisotropy) is almost imperceptible in the lo phase (Figure 5A), which could point to a lack of effect in this phase. Nevertheless, further analysis of the model systems labeled with di-4-ANEPPS has shown that 2OHARA especially affects Chol-rich domains (lo), overriding the Chol effect on the hydration, H-bonding patterns, and membrane dipole potential. As previously hypothesized,[13] this result supports the idea that the FAs interact with phospholipids/sphingolipids, diminishing their interaction with Chol, which can widely affect the molecular interactions within lipid rafts. The results of the present study support the notion that by competing for hydrogen bonds with the sphingolipid headgroup the 2-OHFA will strongly affect sterol/sphingolipid interactions that are responsible for certain particular features of the lo phase such as a large dipole potential, which was seen here to be reduced in the case of 2-OHARA to the same level as in membranes devoid of Chol. Moreover, the establishment of H bonds with a different molecule explains the changes in H-bonding patterns revealed by the spectral shifts in di-4-ANEPPS emission, which are also much stronger for the membranes in the lo phase than in either ld or gel membranes.

■ CONCLUDING REMARKS

The present study aims to understand the biophysical effects of 2OHFA in lipid bilayer membranes. For this purpose, different biophysical techniques and model systems were employed. Overall, the results show that 2OHFA is able to interact spontaneously with the membrane and reorganize the lipid bilayer microdomains in a phase-independent manner.

FA and 2OHFA incorporation into the membrane bilayer occurs spontaneously up to Π values of ∼40 mN/m, above the standard Π values of a cell membrane (∼30 mN/m). This result becomes a direct proof of insertion of 2OHFA and FA into lipid model membranes and suggests similar behavior in living cells. The incorporation of FA and 2OHFA occurs above critical micellar concentrations, which is important, for instance, in understanding cellular effects of these compounds because typical concentrations for cell treatment are on the order of 0.1 μM, attaining a level of 200 μM.

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Thus, we demonstrate that 2OHFA incorporation affects the biophysical properties of the membrane. The strong changes induced by 2OHARA in membrane organization, hydration, and dipole potential suggest that in opposition to the other 2OHFA studied the initial interaction with the membrane and the domain reorganization might be an important step in its mechanism of action.

■ ASSOCIATED CONTENT

Supporting Information

Determination of oxidation of FA or 2OHFA. Di-4-ANEPPS-normalized excitation and emission spectra in gel, ld, and lo single-phase membranes, control, and in the presence of 2OHFA. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

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■ ABBREVIATIONS

Π, surface pressure; 2OHARA, 2-hydroxyarachidonic acid; 2OHDHA, 2-hydroxydocosahexaenoic acid; 2OHEPA, 2-hydroxyeicosapentaenoic acid; 2OHFA, 2-hydroxy fatty acid; 2OHOA, 2-hydroxyoleic acid; ARA, arachidonic acid; Chol, cholesterol; DHA, docosahexaenoic acid; di-4-ANEPPS, 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium hydroxide inner salt; DMSO, dimethylsulfoxide; DPH, 1,6-diphenyl-1,3,5-hexatriene; EPA, eicosapentaenoic acid; FA, fatty acid; FFA, free fatty acid; GUV, giant unilamellar vesicle; ITC, isothermal titration calorimetry; ld, liquid-disordered; lo, liquid-ordered; LUV, large unilamellar vesicle; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; SM, sphingomyelin; *t*-PnA, *trans*-parinaric acid

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SUPPORTING INFORMATION

Changes in membrane organization upon spontaneous insertion of 2-hydroxylated unsaturated fatty acids in the lipid bilayer

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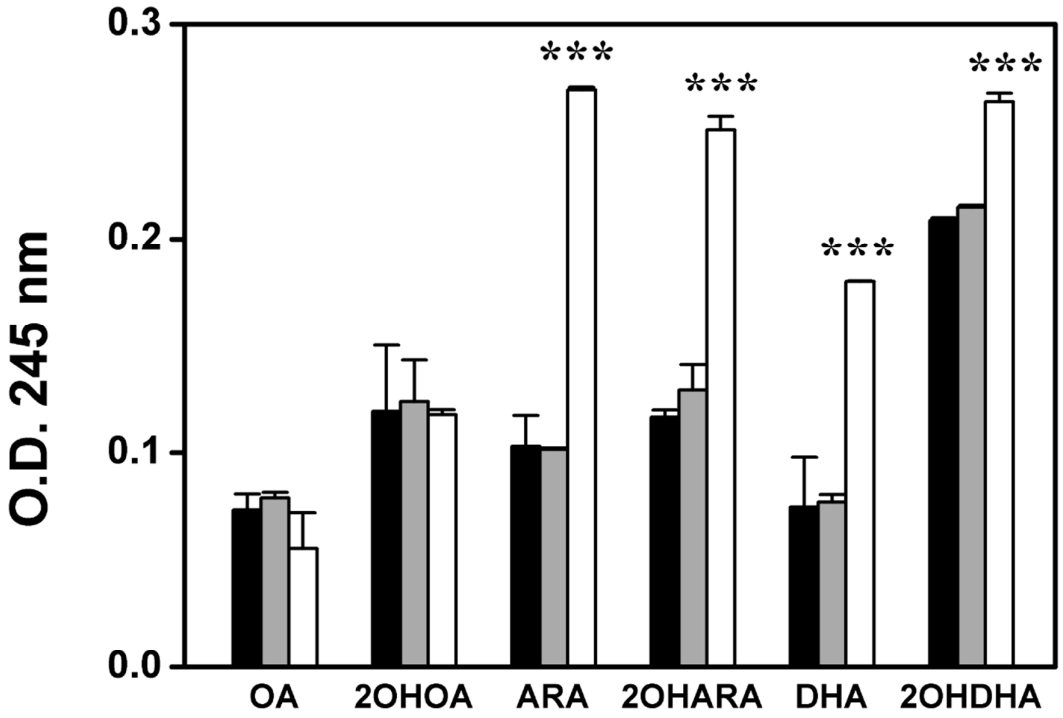

FIGURE S1. Determination of oxidation of FA or 2OHFA. Lipid oxidation was determined by absorption at 245 nm. The negative oxidation control (black bars) was measured by diluting 1 mg/ml OA or 2OHOA and 0.05 mg/ml ARA, 2OHARA, DHA or 2OHDHA in 2 ml of 10 mM Hepes, 1 mM EDTA, 100 mM NaCl, 50 μM butylated hydroxytoluene, pH 7.4. Lipids were immediately extracted by addition of chloroform:methanol (2:1, by vol), following evaporation of the lower organic phase. The lipid film was resuspended in ethanol and absorption at 245 nm was recorded. Lipids were left at room temperature for 60 minutes in the absence of butylated hydroxytoluene to simulate surface pressure experiment conditions (grey bars). As a control of oxidative state, FA or 2OHFA solutions were incubated at room temperature for 24 h in the absence of EDTA and in the presence of 12 μM $CuSO_4$ (empty bars). Data are mean values ± S.D. of 2 independent experiments. *** means $p<0.001$.

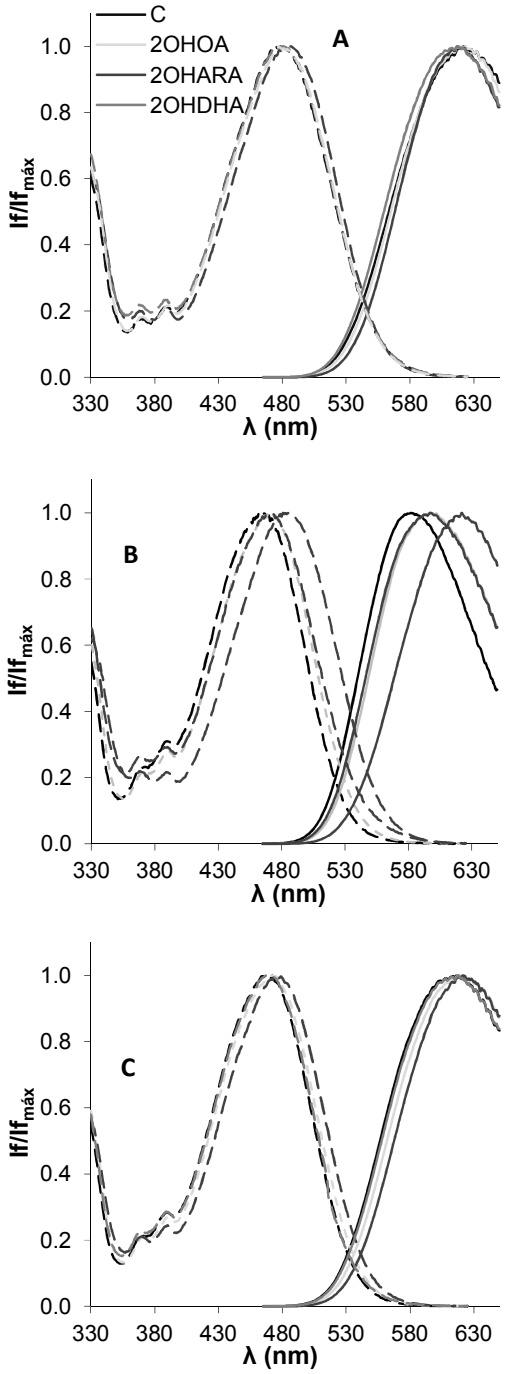

FIGURE S2. Di-4-ANEPSS normalized excitation (λ_{em} = 610 nm) and emission spectra (λ_{exc} = 465 nm) in liposomes with compositions given in Table 1 and corresponding to a single gel **(A)**, l_o **(*B*)** or l_d **(*C*)** phase, and spectral shifts induced by the 2-hydroxylated fatty acids.