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

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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

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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.