Free Radical Biology and Medicine ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Molecular speciation and dynamics of oxidized triacylglycerols in lipid droplets: Mass spectrometry and coarse-grained simulations

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ARTICLE INFO

Article history:
Received 14 May 2014
Accepted 25 July 2014

Keywords:
Lipid droplet
Neutral lipids
Lipid oxidation
Hypoxia
Cancer

ABSTRACT

Lipid droplets (LDs) are ubiquitous and physiologically active organelles regulating storage and mobilization of lipids in response to metabolic demands. Among the constituent LD neutral lipids, such as triacylglycerols, cholesterol esters, and free fatty acids, oxidizable polyunsaturated molecular species may be quite abundant, yet the structural and functional roles of their oxidation products have not been studied. Our previous work documented the presence of these peroxidized species in LDs. Assuming that hydrophilic oxygen-containing functionalities may markedly change the hydrophobic/hydrophilic molecular balance, here we utilized computational modeling to test the hypothesis that lipid peroxidation causes redistribution of lipids between the highly hydrophobic core and the polar surface (phospho)lipid monolayer—the area enriched with integrated enzymatic machinery. Using quantitative liquid chromatography/mass spectrometry, we characterized molecular speciation of oxTAGs in LDs of dendritic cells in cancer and hypoxic trophoblasts cells as two cellular models associated with dyslipidemia. Among the many types of oxidized lipids identified, we found that oxidatively truncated forms and hydroxyl derivatives of TAGs were the prevailing oxidized lipid species in LDs in both cell types. Using coarse-grained molecular dynamics (CG-MD) simulations we established that lipid oxidation changed their partitioning whereby oxidized lipids migrated into the outer monolayer of the LD, where they can affect essential metabolic pathways and undergo conversions, possibly leading to the formation of oxygenated lipid mediators.

Introduction

Lipid droplets (LDs) are dynamic cytoplasmic organelles that can be found in nearly all eukaryotic cells [1,2]. They play important roles during changes in metabolism of intracellular neutral lipids, particularly free fatty acids [3]. Not surprisingly, LDs have been related to dyslipidemias that are characteristic of a number of chronic diseases, including obesity, diabetes, atherosclerosis, and the metabolic syndrome [4–7]. The structural organization and function of LDs are determined by the physico-chemical intermolecular interactions of neutral lipids with each other in the hydrophobic core and with phospholipids, particularly phosphatidylcholine (PC), as well as with proteins embedded in the surface monolayer [1]. The number of PCs and other polar molecules available to cover sufficient surface area of the LD defines its overall size in the aqueous cytosolic environment [8,9]. Moreover, the surface monolayer, along with its enzymatic machinery and corresponding adapter proteins, is viewed as a locale of metabolic conversions of constituting LD lipids [10,11].

Among the neutral lipids comprising the hydrophobic core, triacylglycerols (TAGs), cholesterol esters (ChEs), and free fatty acids (FFAs)—in different proportions—are the most prominent components of LDs [12]. While the composition and molecular

Abbreviations: ChEs, cholesterol esters; CG-MD, coarse-grained molecular dynamics; DCs, dendritic cells; FFAs, free fatty acids; LDs, Lipid droplets; oxTAGs, oxidized TAGs; PHT, primary human trophoblasts; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TAGs, triacylglycerols; TES, tumor explant spleen

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.042
0891-5849/

identities of neutral lipids are influenced by extra- and intracellular metabolic demands, a significant proportion of these molecules may be represented by polyunsaturated species with two or more double bonds [13]. This suggests that the vulnerability to oxygenation may lead to the appearance of polar functionalities in the hydrophobic acyls, shifting the molecular hydrophobic/hydrophilic balance [14], and causing significant modifications of LDs organization. Indeed, we have recently documented the presence of oxygenated TAGs, ChEs, and FFAs in immunologically impaired dendritic cells from tumor-bearing animals and cancer patients [15–17]. Reasoning that these oxidative modifications of polyunsaturated fatty acid (PUFA)-containing LDs may be widespread, here we undertook a combined mass spectrometry (MS) and computational molecular dynamics (MD) study of the effects triggered by the appearance of oxidized TAGs (oxTAGs) in LDs. Given the myriads of possible diversified oxygenated TAGs, our choice was based on the LC-MS-based detection of these species in two types of cells—dendritic cells of tumor-bearing mice and primary human placental trophoblasts—which accumulate LDs as a consequence of hypoxia-induced dyslipidemia [18]. Considering the relatively large total number of atoms in a typical LD that comprises several hundred lipid molecules, coarse-grained molecular dynamics (CG-MD) simulations, rather than full atomistic MD simulations, offer advantages of greater length and time scales for characterizing the dynamic behavior of oxidized lipids [19]. We demonstrate that oxTAGs affect the hydrophobic–hydrophilic balance of LDs, leading to disturbances in phase separation between the neutral lipid core and the phospholipid outer monolayer.

Methods

Mass spectroscopy analysis of LD composition

We used two tumor cell lines, EL4 lymphoma and MC 38 colon carcinomas, maintained in Dulbecco's modified eagle's medium (Invitrogen Corp. Carlsbad, CA) plus 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37 °C, 5% CO_2. DCs were generated from bone marrow progenitor cells using culture with 10 ng/mL recombinant mouse GM-CSF (Invitrogen Corp), and 10 ng/mL interleukin 4 (R&D Systems) as described previously [15]. Tumor explants were prepared from freshly isolated subcutaneous tumors [15]. *In vivo*, dendritic cells (DCs) were purified from spleen of C57BL/6 naïve tumor-free and EL-4 tumor bearing mice using biotinylated CD11c specific antibody (BD Pharmingen) and magnetic beads (Miltenyi Biotec). For *in vitro* generation of DCs, hematopoietic progenitor cells were isolated from bone marrow of tumor-free mice using a lineage cell depletion kit (Miltenyi Biotec) and cultured for 3 days in completed RPMI1640 medium supplemented 10% FBS, antibiotics, and 10 ng/mL granulocyte macrophage colony stimulated factor (Peprotech). Then, the medium was replaced with the one containing 20% v/v tumor explant spleen (TES). After 2 additional days of incubation, DCs were isolated using CD11c antibody and magnetic beads.

Isolation of trophoblast cells and lipid droplets

Primary human trophoblasts (PHT cells) were isolated from healthy singleton term placentas using the trypsin-DNase-dispase/Percoll method as described by Kliman et al., with previously published modifications [20] under an exempt protocol approved by the Institutional Review Board at the University of Pittsburgh. PHT cells were cultured up to 72 h under standard conditions of 20% oxygen. For induction of LD, trophoblasts were cultured between 24 and 72 in hermetically enclosed incubators that supply O_2 $<1\%$, as we previously described [21]. Formation of trophoblast lipid droplets was examined and confirmed using BODIPY 493/503 (Invitrogen/Molecular Probes, Eugene, OR). Isolation of lipid droplets fractions was performed as previously described [22].

Analysis of lipids

Total lipids were extracted from cells by the Folch procedure [23] and analyzed by a Dionex Ultimate 3000 HPLC coupled online to a linear ion trap mass spectrometer (LXQ ThermoFisher). Simultaneous LC/ESI-MS analysis of free fatty acids and their oxidation products was performed as we previously published [17]. The LC/ESI-MS analysis of TAG was performed using gradient solvents as previously described [15,16], including A, methanol, and B, 2-propanol containing 0.1% ammonium hydroxide. The column was eluted during the first 6 min using a linear gradient 0–3% solvent B, from 6 to 18 min isocratic at 3% solvent B, from 18 to 35 min with a linear gradient from 3 to 40% solvent B, then 35–40 min isocratic using 40% solvent B, 40–80 min with a linear gradient from 40 to 55% solvent B, 80–83 min isocratic using 55% solvent B, then 83–85 min with linear gradient 55–0% solvent B, 85–90 min isocratic at 0% solvent B for equilibration of the column. MS spectra were acquired in positive ion mode, using range zoom (500–1000 *m/z*). TAG cations were formed through molecular ammonium adduction ($TAG+NH_4$). Positional analysis of acyl chains in TAG species was performed after CID fragmentation of TAGs [24,25]. The spectra of cholesterol esters (CE) were also acquired in positive ion mode on a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive, ThermoFisher, Inc., San Jose, CA). Phospholipids were analyzed as previously described [26].

To quantitatively assess lipid contents, the following standards were used: oxFFA (9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid, 13-hydroperoxy-9*Z*,11*E*- octadecadienoic acid, 9-hydroxy-10*E*,12*Z*-octadecadienoic acid, 13-hydroxy-9*Z*, 11*E*-octadecadienoic acid, 9-oxo-10*E*,12*Z*-octadecadienoic acid, 13-oxo-9*Z*,11*E*- octadecadienoic acid, 9(10)-epoxy-12*Z*-octadecenoic acid, 9,10-EpOME, 12(13) epoxy-9*Z*- octadecenoic acid), oxidized cholesterol esters, ((±)-9-hydroxy-10*E*,12*Z*-octadecadienoic acid, cholesteryl ester, (±)-9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid, cholesteryl ester; (±)-13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, cholesteryl ester), and oxTAGs (linolein hydroperoxides, hydroxy linoleins) from Cayman Chemicals (Ann Arbor, MI); TAG internal and reference standards from Supelco (Bellefonte, PA), and phospholipids from Avanti Polar Lipids Inc. (Alabaster, AL).

Statistical analysis was performed by either paired/unpaired Student's *t* test or one-way ANOVA. Significance was set at $P<0.05$. The results are presented as mean values ± SD, derived from at least three experiments.

Setup and optimization of CG model of LD

Coarse-grained simulations were based on the Martini force field [17]. In this approach, four heavy atoms are mapped to one CG bead. Tw hundred triacylglycerol molecules with linoleic fatty acids acyl chains were used to construct the LD core, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) to construct the outer monolayer. The CG model of TAG and POPC molecules is illustrated in Fig. S1A. To create the optimized LD model, we investigated the effect of varying the relative ratio between TAGs and phospholipids on the shape of the resulting LD model. Four different POPC:TAG ratios of 1, 2, 3, and 4 were chosen and subjected to simulation (Fig. S1B). The resulting shapes of the LDs with different lipid compositions, as inspected after 50 ns equilibration, are summarized in Fig. S1C.

Please cite this article as: Mohammadyani, D; et al. Molecular speciation and dynamics of oxidized triacylglycerols in lipid droplets: Mass spectrometry and coarse-grained simulations. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.042