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Cholesterol is enriched in the sphingolipid patches on the substrate near nonpolarized MDCK cells, but not in the sphingolipid domains in their plasma membranes☆

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ABSTRACT

Information about the distributions of cholesterol and sphingolipids within the plasma membranes of mammalian cells provides insight into the roles of these molecules in membrane function. In this report, high-resolution secondary ion mass spectrometry was used to image the distributions of metabolically incorporated rare isotope-labeled sphingolipids and cholesterol on the surfaces of nonpolarized epithelial cells. Sphingolipid domains that were not enriched with cholesterol were detected in the plasma membranes of subconfluent Madin-Darby canine kidney cells. Surprisingly, cholesterol-enriched sphingolipid patches were observed on the substrate adjacent to these cells. Based on the shapes of these cholesterol-enriched sphingolipid patches on the substrate and their proximity to cellular projections, we hypothesize that they are deposits of membranous particles released by the cell.

1. Introduction

Plasma membrane function is influenced by the abundances of cholesterol and sphingolipids within mammalian cells, as depletion of these membrane components affects protein distribution and activity. These observations, along with studies of protein recovery in detergent-resistant lipid membranes [1–5] and virus budding from polarized cells [6,7], led to the hypothesis that the plasma membranes of mammalian cells contain domains enriched with cholesterol and sphingolipids [8]. This hypothesis has stimulated much effort to characterize the cholesterol and sphingolipid distributions within the plasma membrane, but these efforts are hampered by the difficulty of directly imaging distinct lipid species in membranes. Although fluorophore-labeled lipids can be incorporated into cells and imaged with fluorescence microscopy, the fluorophore may alter the distribution of the labeled lipid in the plasma membrane by perturbing its intracellular trafficking and interactions with other membrane components [9–12]. Furthermore, fluorescent intensity variations that signify changes in the fluorophore's local concentration are difficult to discriminate from those produced by projections and folds on the cell surface [13,14].

We have previously used an imaging technique that is complementary to fluorescence microscopy, called high-resolution secondary ion mass spectrometry (SIMS), to investigate the organization of specific lipid species in the plasma membranes of individual cells [15–20]. High-resolution SIMS performed with a Cameca NanoSIMS 50 can image rare stable isotope-labeled lipid species in the plasma membrane with a lateral resolution better than 100 nm. Using this approach, we have imaged the distributions of metabolically incorporated isotope-labeled cholesterol and sphingolipids on the surfaces of mouse fibroblast cells (NIH3T3 line) [15,19]. We found that the plasma membranes of these mouse fibroblast cells contained sphingolipid domains that were distinctly different from lipid rafts [15,16]. These sphingolipid domains were not enriched with cholesterol, and cholesterol was instead uniformly distributed within the plasma membrane [15,19]. These observations led us to conclude that favorable interactions between cholesterol and sphingolipids were not responsible for sphingolipid organization in the plasma membranes of mouse fibroblast cells. Based on these results, we also predicted that the plasma membranes of other types of mammalian cells also contain sphingolipid domains that are not enriched with cholesterol.

Here, we begin to test this hypothesis by imaging the distributions of rare stable isotope-labeled sphingolipids and cholesterol (^{15}N-sphingolipids and ^{18}O-cholesterol, respectively) in the plasma membranes of epithelial cells. We focused on epithelial cells from the Madin-

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Darby canine kidney (MDCK) cell line because observations made using this cell line were critical in the development of the lipid raft hypothesis [1,2,6,21,22]. MDCK cells must be grown in continuous monolayers to reach complete polarization [23,24], but in our experience, isolated cells produce the high signal intensities required for sensitive cholesterol and sphingolipid detection with high-resolution SIMS. Therefore, here we used high-resolution SIMS to image the distributions of ^{15}N-sphingolipids and ^{18}O-cholesterol on the dorsal surfaces of subconfluent, nonpolarized MDCK cells and on the adjacent substrate.

2. Materials and methods

2.1. Materials

Trypsin was from Worthington Biochemical Corp. UltraMDCK serum-free medium was purchased from Lonza. Fatty acid-free bovine serum albumin (BSA), and the cell culture materials were purchased from Sigma. Poly-L-lysine and reagents for chemical fixation were purchased from Electron Microscopy Sciences. The 5-mm × 5-mm silicon wafer chips were purchased from Ted Pella Inc. The ^{15}N-sphingolipid precursors, ^{15}N-sphingosine and ^{15}N-sphinganine, were synthesized from ^{15}N-serine that was purchased from Cambridge Isotope Laboratories [25–27]. The ^{18}O-cholesterol was synthesized from i-cholesteryl methyl ether and ^{18}O-water purchased from Sigma and Olinax, Inc., respectively, following published procedures [28]. MDCK cells were purchased from ATCC (CCL-34).

2.2. Cell culture and metabolic labeling

Following receipt from the ATCC, MDCK cells were passaged four times to produce stock cultures. The stock culture was passaged another five times to adapt the cells to UltraMDCK serum-free medium. Three separate batches of metabolically labeled MDCK cells were prepared on different days with the following protocol. MDCK cells adapted for serum-free conditions were cultured in UltraMDCK serum-free medium supplemented with penicillin G, streptomycin, and 3.2 μM ^{15}N-sphingolipid precursors at 37 °C in 5% CO_2. Each day, the cell medium was removed by aspiration and replaced with fresh medium containing ^{15}N-sphingolipid precursors. On the third day, the cells were passaged into UltraMDCK serum-free medium supplemented with antibiotics, 3.2 μM ^{15}N-sphingolipid precursors, and 50 μM ^{18}O-cholesterol (2:5 mass ratio of ^{18}O-cholesterol:fatty acid-free BSA). The medium was aspirated and replaced with fresh medium containing antibiotics, 3.2 μM ^{15}N-sphingolipid precursors and 50 μM ^{18}O-cholestrol every day. On the sixth day, the cells were passaged into dishes that contained poly-L-lysine–-coated silicon wafer chips (5-mm × 5-mm). On the seventh day, the silicon wafer chips with attached cells were washed twice in PBS and fixed with 4% glutaraldehyde and 1% osmium tetroxide, as previously described [15,17,20]. The density of the fixed cells on the substrate that supported representative MDCK cells 1 and 2 is shown in Fig. S1. Up to 50% of the cells detected from the substrate during fixation, so the density of the cells on the substrate prior to fixation was higher than that shown in Fig. S1. The cells that remained adhered to the culture dish were used to measure the incorporation of nitrogen-15 into sphingolipids and oxygen-18 into cholesterol by LC–MS and GC–MS, respectively, as described [15,17].

2.3. SIMS

A total of eight different cells from three separate batches of samples were imaged with high-resolution SIMS during two separate SIMS analyses. Well-preserved cells with normal morphologies were identified by imaging the chips with a JEOL 7000F analytical scanning electron microscope operated at 1 keV and a 10 mm working distance. The samples were coated with iridium (99.95%) as previously described [18], and analyzed using a Cameca NanoSIMS 50 at Lawrence Livermore National Laboratory. The secondary electrons and the $^{16}O^-$, $^{18}O^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, and $^{32}S^-$ secondary ions were collected in parallel. The dorsal surfaces of individual cells were imaged with a 0.17-pA, 16-keV ^{133}Cs + primary ion beam. Images of 15-μm × 15-μm regions were compiled from 12 replicate scans of 512 × 512 pixels that were acquired with a dwell time of 0.5 ms/pixel. Based on the primary ion beam current and sputter time used to acquire these images, and the average sputtering rate reported for other biological materials [29], a sputter depth of approximately 3 nm was calculated.

2.4. Image analysis

To generate isotope enrichment images, LIMAGE software (L. R. Nittler, Carnegie Institution of Washington) run on the PV-Wave platform (Visual Numerics, Inc.) was utilized. The isotope enrichment is the $^{12}C^{15}N^-/^{12}C^{14}N^-$ or $^{18}O^-/^{16}O^-$ ratio divided by their terrestrial standard abundance ratio (0.00367 and 0.002005, respectively). The resulting ratio is the number of times the abundance of the rare isotope-labeled species has increased compared to an unlabeled (natural abundance) sample. The ^{15}N- and ^{18}O-enrichment images were constructed by using a color scale to indicate the isotope enrichment measured at each pixel. A 5 × 5 pixel moving average smoothing algorithm was applied to the isotope enrichment images to reduce random pixel-to-pixel variations characteristic of noise. Additionally, the isotope enrichment was masked at the pixels where the counts of the naturally abundant ion were below a threshold value because dividing by small numbers could magnify any remaining random variations characteristic of noise. The masking threshold for the ^{15}N-enrichment images was 5% of the maximum $^{12}C^{14}N^-$ counts/pixel detected on the cells, which corresponded to four counts of $^{12}C^{14}N^-$ ions/pixel. To remove the variation in the ^{18}O-enrichment characteristic of random noise, the masking threshold was increased to 10% of the maximum $^{16}O^-$ counts/pixel detected on the cells, which was two $^{16}O^-$ counts/pixel. The masked pixels appear black in the isotope enrichment images.

A combination of LIMAGE software run on the PV-Wave platform and the MATLAB Statistics Toolbox were used to calculate the statistically significant thresholds for ^{15}N-enrichment that denote ^{15}N-sphingolipid domains. Briefly, LIMAGE was used to divide the cell surface into 5 × 5 pixel regions of interest (ROIs), where the $^{12}C^{14}N^-$ counts detected at each pixel within the ROI was at least 5% of the maximum detected on the cell. The ^{15}N-enrichments were calculated for each ROI, exported from LIMAGE, and tabulated for each cell. To determine the mean ^{15}N-enrichment and 1 SD for the regions on the cell that did not contain sphingolipid domains, the ^{15}N-enrichments measured in all 5 × 5 pixel ROIs on the MDCK cell of interest were imported into MATLAB, and a frequency distribution was constructed of these ^{15}N-enrichment values. Then, the MATLAB Statistics Toolbox was used to simulate the probability distribution function for a mixture of two normal distributions that best fit the distribution of measured ^{15}N-enrichment values. The mean and standard distribution (SD) of each of the two distributions in the best-fit mixture model was recorded. The distribution with the smaller mean corresponds to the regions of the plasma membrane that lacked ^{15}N-sphingolipid domains, and the distribution with the higher mean represented the ^{15}N-sphingolipid domains in the plasma membrane. The statistically significant threshold for the ^{15}N-enrichment, which denotes statistically significant ^{15}N-sphingolipid enrichment, was defined as the ^{15}N-enrichment that was at least 2 SD above the mean ^{15}N-enrichment for the domain-free regions on the cell. For representative cell 1, the analysis of 29,614 ROIs on the cell yielded a mean ^{15}N-enrichment = 3.3 and 1 SD = 1.04, so ^{15}N-enrichments > 5.4 were statistically significant. For representative cell 2, the analysis of 24,960 ROIs on the cell yielded a mean ^{15}N-enrichment = 3.8 and 1 SD = 1.51, so ^{15}N-enrichments > 6.8 were statistically significant.

Next, LIMAGE software was used to define the regions on the cell

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