Biochemistry and Biophysics Reports 10 (2017) 132–136

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Characterization of lipid rafts in human platelets using nuclear magnetic resonance: A pilot study

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ARTICLE INFO

Keywords:
NMR
Platelets
Rafts
Lipid microdomains

ABSTRACT

Lipid microdomains ('lipid rafts') are plasma membrane subregions, enriched in cholesterol and glycosphingolipids, which participate dynamically in cell signaling and molecular trafficking operations. One strategy for the study of the physicochemical properties of lipid rafts in model membrane systems has been the use of nuclear magnetic resonance (NMR), but until now this spectroscopic method has not been considered a clinically relevant tool. We performed a proof-of-concept study to test the feasibility of using NMR to study lipid rafts in human tissues. Platelets were selected as a cost-effective and minimally invasive model system in which lipid rafts have previously been studied using other approaches. Platelets were isolated from plasma of medication-free adult research participants ($n=13$) and lysed with homogenization and sonication. Lipid-enriched fractions were obtained using a discontinuous sucrose gradient. Association of lipid fractions with GM1 ganglioside was tested using HRP-conjugated cholera toxin B subunit dot blot assays. ^{1}H high resolution magic-angle spinning nuclear magnetic resonance (HRMAS NMR) spectra obtained with single-pulse Bloch decay experiments yielded spectral linewidths and intensities as a function of temperature. Rates of lipid lateral diffusion that reported on raft size were measured with a two-dimensional stimulated echo longitudinal encode-decode NMR experiment. We found that lipid fractions at 10–35% sucrose density associated with GM1 ganglioside, a marker for lipid rafts. NMR spectra of the membrane phospholipids featured a prominent 'centerband' peak associated with the hydrocarbon chain methylene resonance at 1.3 ppm; the linewidth (full width at half-maximum intensity) of this 'centerband' peak, together with the ratio of intensities between the centerband and 'spinning sideband' peaks, agreed well with values reported previously for lipid rafts in model membranes. Decreasing temperature produced decreases in the 1.3 ppm peak intensity and a discontinuity at ~18 °C, for which the simplest explanation is a phase transition from L_d to L_o phases indicative of raft formation. Rates of lateral diffusion of the acyl chain lipid signal at 1.3 ppm, a quantitative measure of microdomain size, were consistent with lipid molecules organized in rafts. These results show that HRMAS NMR can characterize lipid microdomains in human platelets, a methodological advance that could be extended to other tissues in which membrane biochemistry may have physiological and pathophysiological relevance.

1. Introduction

Lipidomics has wide implications for human health. Among the most fundamental roles for lipids is their participation in cell membranes, where they serve not only structural but also dynamic functions. Current understanding of cell membrane organization postulates the existence of lipid microdomains, or rafts, comprised principally of cholesterol and sphingolipids, which self-segregate from the surrounding phospholipid bilayer [1] due to sterically aversive forces [2]. The largely dietarily-determined lipid balance can affect the physicochemical properties of the membrane microdomains, with downstream effects on cell-cell signaling, molecular trafficking, and regulation of transmembrane proteins, including a variety of transporters and G-protein coupled receptors [3]. Among technologies used to study the composition of lipid rafts, ^{1}H detected high-resolution magic-angle spinning nuclear magnetic resonance (HRMAS NMR) utilizes the

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http://dx.doi.org/10.1016/j.bbrep.2017.03.005
Received 22 December 2016; Received in revised form 28 February 2017; Accepted 15 March 2017
Available online 20 March 2017
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natural proton signals from lipids to provide very good resolution of chemically shifted resonances through rapid spinning of samples at the "magic angle" (54.7°) with respect to the main magnetic field. NMR has been used to characterize lipid rafts in model membrane systems [4–9,11–14], in cell culture [15–17], and in the influenza viral envelope [18]. However, NMR has not been utilized in clinical studies of human cell membranes. Therefore we proceeded to test the feasibility of using HRMAS NMR to characterize lipid rafts in human platelets, in which lipid rafts have been successfully studied using other methods [19–22]. The NMR approach allows for physicochemical characterization of lipid rafts at the molecular level. Potential applications include studies of the effects of dietary intake of lipids (e.g., polyunsaturated fatty acids and cholesterol) and antihyperlipidemic medications on cell membrane functioning in healthy individuals and in pathological states.

2. Materials and methods

2.1. Sample

Platelets were isolated from plasma of medication-free adult research participants ($n=13$) with major depressive disorder who were otherwise medically healthy, and who had enrolled in mood disorders protocols at the New York State Psychiatric Institute and gave informed consent to the collection of blood samples for biochemical studies.

2.2. Purification of platelet lipid domains

Platelets were extracted from blood samples as previously described according to methods optimized by our research team [23]. Briefly, the blood was collected in EDTA vacutainer tubes and centrifuged at low speed to obtain the platelet-rich-plasma, which was then further centrifuged (12,000 × *g*, 4 °C, 4 min). The resulting pellets were resuspended in 5 mL of ice-cold phosphate-buffered saline (PBS, pH 7.4) and again pelleted and stored at −80 °C until used. For the next (homogenization) step, the platelet pellets were resuspended and washed twice in ice-cold PBS and suspended in 2 mL of 500 mM Na_2CO_3 solution, pH 11.0.

To avoid artifacts associated with detergent extraction [24,25], lipid rafts were isolated from extracted platelets using non-detergent methods: cells were lysed using a Polytron Homogenizer (Brinkmann, Lucerne, Switzerland; three 10-s bursts at setting 3) and then subjected to sonication with an XL-2000 Ultrasonic Cell Disruptor (Microson, Newtown, CT, USA; three 20-s bursts at setting 5). The homogenate was then adjusted to 45% sucrose by adding 2 mL of 90% sucrose prepared in 25 mM MES-buffered saline (MBS; 25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 0.15 M NaCl). The fraction enriched in lipids was isolated by a 5–35% discontinuous sucrose gradient [5%, 15%, 25%, 35%], followed by ultracentrifugation (100,000 × *g* for 20 h at 4 °C) with flotation (adapted from [26]). Fractions containing lipids exhibited visible opalescence after ultracentrifugation (Fig. 1A). The interface was removed, diluted with 2X MBS buffer, and centrifuged (10,000 rpm for 20 min); the pellets were saved and stored at −20 °C for subsequent lipid extraction. The presence of GM1 gangliosides in the lipid fraction was assessed with GM1-specific horseradish peroxidase (HRP)-conjugated cholera toxin B subunit dot blot assays [27] using chemiluminescence (Fig. 1B). The quantity of proteins in lipid and non-lipid fractions was determined by Bicinchoninic (BCA) Protein Assay (Thermo Fisher Scientific; Rockford, IL, USA) [28] and spectrophotometry (Fig. 1C). The Folch method [29] was used to extract the lipids from the sucrose solution into an 8:4:3 chloroform-methanol-water mixture. Lipid samples were lyophilized and stored at −80 °C.

2.3. Lipid ^{1}H HRMAS NMR spectra

To prepare samples for NMR, the frozen lyophilized lipids were

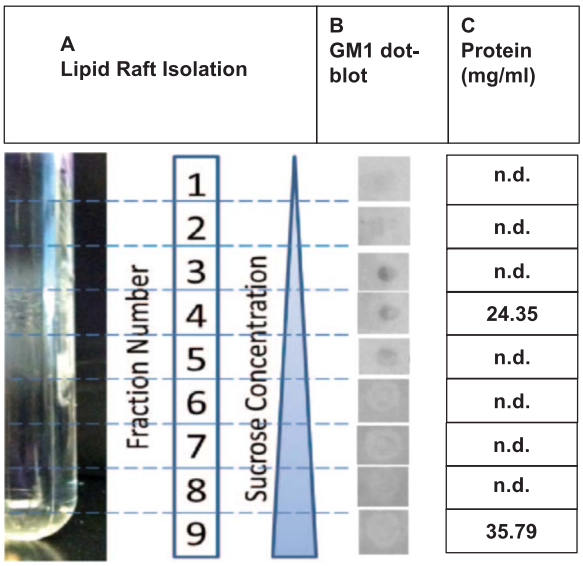

Fig. 1. Purification and validation of lipid rafts from human platelets. **A.** Visible opalescent lipid band with respect to the sucrose fractionation. **B.** GM1-specific HRP-conjugated cholera toxin B subunit dot blot assays. **C.** BCA assay differentiates between lipid-associated (fraction 4) and lipid non-associated (fraction 9) proteins; 'n.d.' indicates no protein detected.

reconstituted in deuterated water (D_2O). Five washes in D_2O were each performed by adding 300 μL of deuterated water to each sample in a microcentrifuge tube, spinning the samples at 10,000 rpm for 15 min, and decanting the clear layer using a pipette. Between each wash, samples were frozen via direct contact of the outside of the microcentrifuge tube with dry ice and thawed at room temperature followed by gentle vortexing. These freeze-thaw cycles were performed in order to facilitate H/D exchange between the solvent and membrane fragments. Samples thus prepared for HRMAS NMR were slightly viscous and visibly, uniformly opaque suspensions of multibilayers.

Butylated hydroxytoluene (BHT) was added to all samples to prevent oxidation of the unsaturated lipid acyl chains. Water-soluble 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added to all samples at a ratio of 100:1 lipid:DSS by weight as a chemical shift calibration standard for NMR spectroscopy.

NMR data were acquired at the New York Structural Biology Center on a 4-channel Bruker Avance I widebore spectrometer equipped with a 4 mm HRMAS HCND probe and ZrO_2 rotors (Bruker Biospin, Karlsruhe, Germany), operating at a ^{1}H resonance frequency of 750 MHz and using a spectral width of 15 kHz. Samples were spun at an angle of 54.7° to the magnetic field direction (the 'magic angle') at spinning frequencies of 4–5 kHz to obtain well-resolved spectra. ^{1}H spectra were acquired with a single-pulse Bloch decay experiment [30] using an 8 μs 90° pulse. We acquired 128 scans with a recycle delay of 2 s between data acquisitions, thus requiring ~4 min to obtain each spectrum. The signal-to-noise ratio for the main lipid resonance was 50:1, allowing for reliable estimates of peak intensity and linewidth at half height. A presaturation water suppression technique was used to suppress the signal from residual ^{1}H nuclei in the D_2O solvent. Experiments were executed with and without a pre-acquisition echo sequence to test for the presence of broad signals [31]. Measurement temperatures were varied from −10 °C to 35 °C and calibrated with a methanol standard [32]. Sideband / centerband intensity ratios and linewidth differences of the principal lipid resonance at ~1.3 ppm were monitored *vs.* temperature, and these ratios were used to validate the presence of rafts in the L_o phase [13].

Rates of lipid lateral diffusion (signal intensity decay as a function of time) were measured in a two-dimensional stimulated echo longitudinal encode-decode experiment conducted with presaturation and bipolar gradients [33] (256 scans, gradient length 8 μs, recycle delay

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