^{1}H NMR spectroscopy analysis of isolated intracellular lipid droplets from a human cancer cell line, BE(2)M17

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Introduction

^{1}H NMR spectroscopy allows direct detection and quantification of specific lipid species in situ. The origin of these lipid signals was first thought to be from globular plasma membrane micro-domains, but more recent direct evidence suggests that the NMR signals are most likely to be from mobile lipid droplets (LDs) in the cytoplasm. The lipid signals in NMR spectra from cell extracts are usually dominated by membrane lipids which can mask the signals from sub-cellular compartments, such as LDs. It is therefore desirable to isolate the LDs in order to eliminate the signals from membrane lipids. This paper presents the ^{1}H NMR spectra acquired from isolated LDs of a human neuroblastoma cell line and provides a new method to investigate these highly dynamic organelles separated from tumour cells.

Methods

HR-MAS

HR-MAS was performed on a Varian 600-MHz (14.1 T) vertical bore spectrometer using a 4-mm gHX nanoprobe (Varian NMR Inc) with a three channel Inova console running VNMRj software. The probe temperature was set to 0.1^{0}C, which equated to a temperature inside the rotor of 6.7 ^{0}C determined by calibration using methanol with a rotor speed of 2500 Hz. The pulse sequence used for lipid investigation consisted of a single 90^0 pulse with a 1s duration water presaturation pulse.

Isolation of LDs

Cells were ground in deionised water or D_2O and the homogenate was centrifuged at 2000g at 4^{0}C for 10 min. The supernatant was adjusted to 18.46% sucrose, topped with the same volume of deionised water or D_2O and centrifuged at 142,000g for 90min at 4^{0}C. After ultracentrifugation, the sample was separated into three fractions and only the top isolated fraction was collected for extraction and subsequent NMR analyses.

Staining of LDs

Saturated Oil Red O in 70% ethanol was applied to BE(2)M17 cells or isolated fraction for 20 min. Slices washed in running water and then followed by haematoxylin staining. For Nile red staining, cells were resuspended in 1µg/ml Nile red for 5 min under dark condition. Negative staining was performed on the electron microscopy grid with the isolated fraction and then observed using JEOL 1200EX TEM. LD sizes (n>50) were measured using ImageJ and presented as mean value ± standard deviation.

Liquid-state ^{1}H NMR spectroscopy

A dual phased methanol-chloroform extraction protocol was used to prepare the lipids for NMR analysis. Lipid extracts were resuspended in 600µl deuterated chloroform containing 0.03 % (v/v) TSP. ^{1}H NMR spectra of cell extracts and the isolated LDs suspended in D_2O were recorded on a Varian 600-MHz (14.1 T) vertical bore spectrometer using a HCN probe. A standard pulse and acquire sequence was used which consisted of a single 90^0 pulse preceded by one second of water presaturation. At least three independent preparations were used for isolation and extraction.

Results and Discussion

Lipid droplets from BE(2)M17 cells

Oil red O and haematoxylin staining (Figure 1a) and Nile red staining (Figure 1b) illustrated that there were a considerable number of cytoplasmic LDs inside the cells. The average size of these LDs with Oil red O staining inside BE(2)M17 cells was 0.34±0.089µm whereas in the isolated fraction it was 0.22±0.054µm. The LDs in the isolated fraction (Figure 1c) had a distribution of sizes ranging from 0.1-0.5 µm in diameter. Membrane bound vesicles can be visualised with oil red O and haematoxylin staining, appearing as annular structures easily distinguishable from LDs. Figure 1c shows membrane bound vesicles were absent from the isolated fraction and the middle sucrose fraction although they could be visualized in other fractions (data not shown). In addition, the transmission electron microscopy data (Figure 1d) shows the existence of LDs and the absence of membrane bound vesicles in the isolated fraction, in accordance with Figure 1c.

^{1}H NMR spectra of lipids

Figure 2 shows the liquid-state ^{1}H NMR spectrum acquired directly from the isolated LDs together with the HR-MAS spectrum of intact cells. The region between 0.5ppm to 3.5ppm has been plotted to exclude signals from sucrose. Metabolites and lipid peaks were observed in both spectra and the lipid peaks were assigned according to previously published value.

Spectra from extracts show a clear separation between the cholesteryl ester and cholesterol peaks at 1.01 and 0.99ppm. A higher ratio of cholesterol ester to cholesterol was present in the isolated LDs (0.81, n=4) compared to the whole cell extract (0.19, n=3) and there is a statistically significant difference between the two groups (P =0.0025).

Conclusion

An excellent agreement was observed between the lipid resonances present in the HR-MAS of BE(2)M17 cells and the NMR spectra of their isolated LDs, strongly supporting that NMR visible lipid resonances originate primarily from LDs. A comparison between the ^{1}H NMR spectra of lipid extracts from the isolated LDs and the whole cell preparations revealed a number of similarities, particularly in the presence of unsaturated lipids. However, differences were also detected, in particular a large dissimilarity was seen in the ratio between the cholesteryl ester and cholesterol peaks.

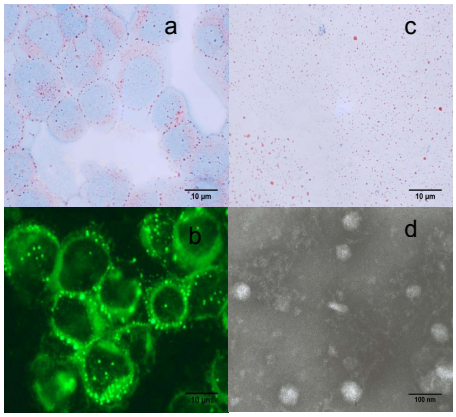

Figure 1. Oil red O and haematoxylin staining a) and Nile red staining b) of BE(2)M17 Oil red O and haematoxylin staining c) and Negative staining d) of isolated fraction.

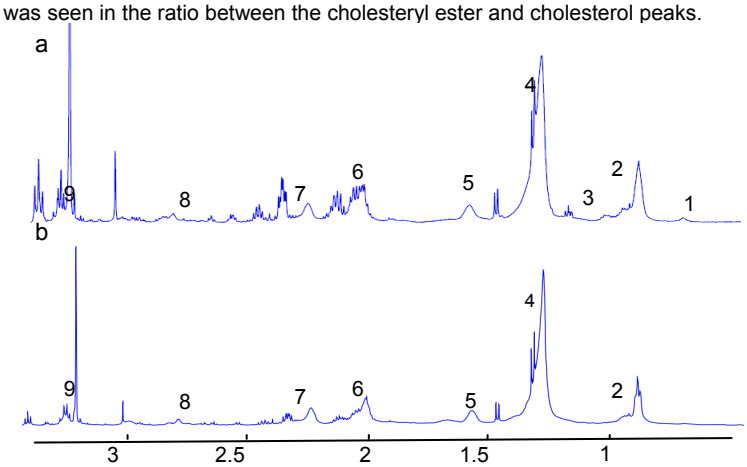

Figure 2. (a) Liquid-state ^{1}H NMR spectrum from the isolated lipid droplet fraction and (b) HR-MAS spectrum from intact cells. 1 C**H**3(Ch,ChE), 2 C**H**3(L,Ch), 3 C**H**3(Ch,ChE),C**H**2(Ch,ChE) 4 C**H**2(L,Ch), 5 C**H**2CH2CO(L), 6 C**H**2CH=CH(L), 7 C**H**2COO(L), 8 =CHC**H**2CH=, 9 N(C**H**3)3 (PtdCho) Ch, cholesterol; ChE, cholesteryl ester Cho, choline residue; L, lipid ; PtdCho, phosphatidylcholine.