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Cholesterol homeostasis in T cells. Methyl-β-cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions

Saleemulla Mahammad, Ingela Parmryd *

Department of Cell Biology, The Wenner-Gren Institute, Stockholm University, 106 91 Stockholm, Sweden

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ABSTRACT

Methyl-β-cyclodextrin (MBCD) is frequently used to acutely deplete cells of cholesterol. A widespread assumption is that MBCD preferentially targets cholesterol in lipid rafts and that sensitivity to MBCD is proof of lipid raft involvement in a cellular process. To analyse any MBCD preference systematically, progressive cholesterol depletion of Jurkat T cells was performed using MBCD and [^{3}H]-cholesterol. It was found that at 37 °C, MBCD extracts similar proportions of cholesterol from the Triton X-100 resistant (lipid raft enriched) as it does from other cellular fractions and that the cells rapidly reestablish the relative differences in cholesterol concentration between different compartments. Moreover, cells restore the cholesterol level in the plasma membrane by mobilising cholesterol from intracellular cholesterol stores. Interestingly, mere incubation at 0 °C caused a loss of plasma membrane cholesterol with a concomitant increase in cholesteryl esters and adiposomes. Moreover, only 35% of total cholesterol could be extracted by MBCD at 0 °C and was accompanied by a complete loss of plasma membrane and endocytotic recycling centre filipin staining. This study clearly shows that MBCD does not specifically extract cholesterol from any cellular fraction, that cholesterol redistributes upon temperature changes and that intracellular cholesterol stores can be used to replenish plasma membrane cholesterol.

1. Introduction

Cholesterol is the most abundant sterol in mammalian membranes and its levels are under rigid control by complex mechanisms [1]. Due to its small polar headgroup and large hydrophobic moiety, cholesterol associates with phospholipids in order to avoid unfavourable exposure to water [2]. The plasma membrane of mammalian cells contains several types of nanodomains of which lipid rafts, in themselves a mixture of small domains with similar properties, are thought to form by the self-aggregation of cholesterol and sphingolipids [3]. Lipid rafts are believed to exist in a liquid ordered (lo) like state that resembles both the liquid disordered state, in that the lipids are fluid, and the gel state, in that the lipids are highly organised. Lipid rafts have become an attractive explanation for processes as diverse as the differential sorting of proteins in epithelial cells, immunological signalling and the entry of pathogens into host cells.

Cholesterol depletion is a popular tool in lipid raft research, based on the idea that cholesterol is an absolute requirement for lipid raft integrity and that its depletion leads to lipid raft dispersion, i. e. if a cellular process is affected by cholesterol depletion it is assumed that lipid rafts are involved. Various methods to lower cholesterol levels have been employed; the culture of cells in the absence of exogenous cholesterol [4], inhibition of cholesterol biosynthesis by statins [5], oxidation of cholesterol [6] and the use of cholesterol binding agents such as digitonin, filipin and saponin [7,8]. The most commonly used method is acute cholesterol depletion using methyl-β-cyclodextrin (MBCD). Unlike the cholesterol binding agents mentioned above that incorporate into membranes, MBCD has a central cavity able to form a 2:1 complex with cholesterol [9,10] and it acts strictly at the membrane surface.

It is a common misconception that MBCD preferentially extracts cholesterol from lipid rafts and it even gets referred to as a lipid raft inhibitor [11–14]. However, cholesterol is not confined to lipid rafts and it is debatable whether it is even enriched in these domains. The phase diagram of the lipid mixture cholesterol:sphingomyelin: dioleylphosphatidyl-choline demonstrates that the difference in cholesterol concentration between lo-domains and ld-domains is quite small [15]. A recent study showing that dihydroergosterol (DHE) is evenly distributed in the plasma membrane of macrophages and hepatoma cells suggests that there is no big difference in cholesterol concentration in different membrane domains also in cells [16]. There is no compelling reason why MBCD should specifically extract cholesterol from lipid rafts. On the contrary, it seems likely that cholesterol would actually be preferentially removed from other areas in the plasma membrane, where the lipids are less tightly packed.

The bulk of free cellular cholesterol, 65–90%, has been reported to reside in the plasma membrane, based on subcellular fractionation or cholesterol oxidase accessibility (reviewed in [17]). However, a study with the fluorescent cholesterol analogue DHE has demonstrated that a substantial cholesterol fraction, 35%, is found in the endocytic recycling compartment (ERC) [18]. In addition, ER membranes contain about

* Corresponding author. Tel.: +46 8 16 39 03; fax: +46 8 15 98 37.
E-mail address: ingela.parmryd@cellbio.su.se (I. Parmryd).

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1.5% cholesterol whereas in the plasma membrane [19], about 30–40% of the lipids are cholesterol. The ratio between the plasma membrane and intracellular membranes differs considerably between cell types, but typically the plasma membrane makes up no more than 5% of the total cellular membrane and the ER accounts for a much larger percentage [20,21]. Clearly this is incompatible with the bulk of the free cholesterol being in the plasma membrane and there is a need for new methods to reinvestigate this issue.

In this study, the distribution of cholesterol in Jurkat T cells subjected to progressive cholesterol extraction with MBCD was addressed. The results presented stress that cells maintain differences in cholesterol levels between cellular compartments by redistributing their remaining cholesterol. Furthermore, upon cold stress cells rearrange their cholesterol which can be quantified to estimate the proportion of cholesterol present in the plasma membrane at physiological temperature.

2. Materials and methods

2.1. Tissue culture

E6.1 Jurkat T cells from ATCC were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) in a humidified incubator under 5% CO_2.

2.2. Materials

CT-B-Alexa 594, anti-rabbit-Alexa 488 and avidin-Alexa 488 were from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-transferrin receptor (TfR) was from MorphoSys (Kingston, NH). [^{3}H]-cholesterol was from Amersham Biosciences (GE Healthcare, Bucks, UK), EN^{3}HANCE spray from Perkin Elmer (Waltham, MA) and TLC-plates from Merck KGaA (Darmstadt, Germany). Sulfo-NHS biotin was from Pierce biotechnologies (Rockford, IL). Unless otherwise stated, chemicals were from Sigma (St Louis, MO).

2.3. Labelling Jurkat T cells with [^{3}H]-cholesterol

2×10^6 cells were washed twice with serum-free medium. Cells were grown for up to 40 h in the presence of 10 μCi [^{3}H]-cholesterol (specific activity 40–42 Ci/mmol) in complete RPMI except that the FCS concentration was 2.5%. Cell aliquots were subjected to liquid scintillation counting after washing in serum-free medium twice. Cells were lysed in chloroform:methanol:water (1:1:0.3 v/v) in which two phases were induced by changing it to chloroform:methanol:water (3:2:1 v/v). The organic phase was evaporated, the residue dissolved in a small volume of chloroform and then loaded on a silica column. Neutral lipids were eluted with chloroform and analysed by TLC on Silica plates developed with chloroform:acetone (49:1 v/v) or by reversed phase HPLC on a Hewlett Packard Hypersil ODS column (pore size 3 μm) using a linear gradient from methanol: water (9:1) to methanol:2-propanol:*n*-hexane (2:1:1). The radioactivity of the eluate was monitored by a radioactivity flow detector (Radiometric Instruments, Tampa, FL). The TLC plate was sprayed with EN^{3}HANCE and the plate was exposed to photographic film for 48 h at −80 °C.

2.4. Determination of cholesterol and cholesteryl esters

2×10^6 cells were washed and extracted as above. The water phase was then extracted with chloroform:methanol (3:1 v:v). The organic phases were pooled, evaporated and the tubes placed at 110 °C 20 min. The residue was dissolved in 200 μl assay buffer (0.1 M potassium phosphate pH 7.4, 50 mM NaCl, 5 mM cholic acid and 0.1% (w/v) Triton X-100 (TX)) and incubated at 37 °C 2 h. Cholesterol and cholesteryl esters were determined using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) [22,23]. 10 μl aliquots were incubated with 2 U/ml HRP and cholesterol oxidase, 300 μM Amplex red ±0.2 U/ml cholesterol esterase at 37 °C. Fluorescence was read after 150 min with excitation at 544 nm and emission at 590 nm in a Fluoroskan II (Labsystems, GMI, Ramsey, MN).

2.5. Depletion of cellular cholesterol

Labelled cells were washed twice with serum-free medium and mixed with non-labelled cells at a ratio of 2:3. Cells were left to equilibrate for 45 min at 37 °C in complete RPMI supplemented with 2% BSA and were then washed twice with serum-free medium. MBCD was dissolved freshly before each experiment and the cell density was kept constant at 10×10^6 cells/ml. Cells at 37 °C were treated with MBCD in the 0.5–15 mM range. At the indicated times, the cells were pelleted by brief centrifugation and the MBCD-containing supernatant was transferred to a fresh tube. The cell pellet was resuspended in PBS and aliquots from both fractions were subjected to scintillation counting. Two protocols resulting in 10%, 20%, 30%, 40% and 50% cholesterol extraction at 37 °C were used for further experiments. In the first protocol, cholesterol was extracted with 2.5 mM MBCD for 2.5, 5.5, 9 and 14.5 min or with 5 mM MBCD for 5 min. In the second protocol, cholesterol was extracted for 15 min with 0.5, 1, 1.2, 2.5 and 3 mM, respectively. Cells at 0 °C were treated with MBCD in the 30–120 mM range.

2.6. Drug treatment

Cells were treated with the HMG-CoA reductase inhibitor simvastatin at 20 μM at a cell density of 2×10^6/ml for 15 min prior to and during MBCD extraction. Simvastatin was also present during the recovery period.

2.7. Cellular fractionation

Labelled cells were mixed with non-labelled cells at a ratio of 2:23 and washed twice with serum-free medium. Cells were treated with MBCD to achieve the desired cholesterol depletion after which they were pelleted and washed in PBS. Triton X-100 detergent resistant membranes (TX-DRMs) were prepared as described previously from 50×10^6 cells lysed for 15 min on ice in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 2 mM EDTA) containing 1% TX, protease inhibitors (5 μg/ml each of antipain, leupeptin, chymostatin and pepstatin and 1 mM PMSF), 5 mM NaF and 1 mM Na_3VO_4. [24]. The sucrose density gradient was made up of 2 ml 40% sucrose, 2 ml 30% sucrose and 1 ml 5% sucrose, all in MNE. TX-DRMs were collected from the 5–30% sucrose interface and pelleted by centrifugation at 100 000 ×*g* for 1 h. The resulting TX-DRM pellet was rinsed and suspended in MNE as was the pellet from the sucrose density gradient tube. The bottom 1.5 ml of the gradient was named the TX-soluble fraction and the 1.5 ml above the intermediate fraction. The resuspended pellets and aliquots of the other fractions were subjected to liquid scintillation counting. In another set of experiments, ten fractions of 500 μl were collected from the bottom of the tube were subjected to liquid scintillation counting.

2.8. FACS analysis

Control cells and cells treated with MBCD were stained with 2.5 μg/ml CT-B-Alexa 594 in PBS containing 2% BSA for 30 min at rt at a cell density of 5×10^6/ml. The cells were analysed on a FACSCalibur (BD Biosciences, San José, CA) with excitation at 595 nm and a 660/8 emission filter. Cells depleted of 50% cholesterol were left to recover in serum-free media for 40 min at 37 °C after which they were fixed for 15 min in 4% PFA. The cells were stained with filipin and washed as above and analyzed on a LSRII (BD Biosciences, San José, CA) with excitation at 358 nm and a 461/50 nm excitation filter as were untreated cells. 10 000 cells from each sample were analysed.

2.9. Filipin staining

For fluorescence microscopy experiments, cells were attached to TESPA-coated coverslips by incubation at 37 °C for 10 min or on ice for 45 min (2.5×10^5 cells/coverslip). Cholesterol extractions at the respective temperatures were performed at the same cell to MBCD ratios as above. Cells were fixed in 4% paraformaldehyde/PBS either at 37 °C for 15 min or at 0 °C for 60 min. A filipin stock of 20 mg/ml DMSO was stored under argon gas at −20 °C. Cells were stained with 50 μl of 50–250 μg/ml filipin at rt for 60 min. Cells were washed three times with PBS and mounted in AF1 (Citifluor Ltd, UK).

2.10. Surface biotinylation

5×10^6 cells were washed with ice-cold PBS, resuspended in 1 mg/ml sulfo-NHS biotin/PBS and incubated at 0 °C for 30 min. The cells were then washed in 100 mM glycine/PBS three times and attached to TESPA-coated coverslips (2.5×10^5 cells/coverslip). The cells were incubated at 37 °C for 10 min or at 0 °C for 20 min and fixed in 4% paraformaldehyde/PBS at the respective temperatures. The cells were stained with 10 μg/ml avidin-Alexa 488 for 30 min at rt.

2.11. Cell viability

Cell viability was assessed by trypan blue exclusion. For each population 300 cells were counted and each experiment was repeated three times.

2.12. Imaging

Wide-field fluorescence microscopy was performed with a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with a AxioCam MRm camera. A 63X oil objective lens (NA 1.4) and DG4 (Sutter Instrument, Novato, CA) with excitation filters 350/50, 480/20 or 565/25 and emission filters 460/50, 525/50 or 645/75 were used for filipin, Alexa-488 and Nile red, respectively. DG4 excitation enabled the acquisition of high quality images of filipin, which is prone to rapid photobleaching when excited with most light sources. To avoid bleaching affecting the image analysis, focus was adjusted under transmitted light and filipin images immediately acquired when the excitation source was turned on. Imaging was performed at the equatorial plane of the cells. Each experiment was repeated at least twice and more than 100 cells meeting the criteria were visually examined from each population. The images were acquired blindly to minimise operator bias. Displayed images were prepared using Adobe Photoshop 7.1 software.

2.13. Estimation of filipin in the plasma membrane

The plasma membrane was delineated manually with sequentially marked points that were joined automatically [25]. Once delineated, the mean fluorescence intensity