Essentially All Excess Fibroblast Cholesterol Moves from Plasma Membranes to Intracellular Compartments

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Abstract

It has been shown that modestly increasing plasma membrane cholesterol beyond its physiological set point greatly increases the endoplasmic reticulum and mitochondrial pools, thereby eliciting manifold feedback responses that return cell cholesterol to its resting state. The question arises whether this homeostatic mechanism reflects the targeting of cell surface cholesterol to specific intracellular sites or its general equilibration among the organelles. We now show that human fibroblast cholesterol can be increased as much as two-fold from 2-hydroxypropyl-β-cyclodextrin without changing the size of the cell surface pool. Rather, essentially all of the added cholesterol disperses rapidly among cytoplasmic membranes, increasing their overall cholesterol content by as much as five-fold. We conclude that the level of plasma membrane cholesterol is normally at capacity and that even small increments above this physiological set point redistribute essentially entirely to intracellular membranes, perhaps down their chemical activity gradients.

Introduction

Sterols are inhomogeneously distributed among the membranes of eukaryotic cells by unknown mechanisms [1–5]. PMs (plasma membranes) are characteristically sterol rich; in some animal cells, they contain ~80% of the total unesterified cell cholesterol at a CH/PL (cholesterol/phospholipid) mole ratio of 0.7–0.8 [6–8]. In contrast, the ER (endoplasmic reticulum) in some animal cells contains on the order of 1% of cell cholesterol at a CH/PL mole ratio of ~0.05 [9–11]. Cell cholesterol is not static but moves between organelles on a timescale of minutes [9,12–15]. A number of proteins in yeast and animal cells have been implicated in this intracellular flux, but it is not clear if any of them apportion sterol molecules to specific intracellular sites or pump sterols against their chemical activity gradients [13,16–21]. Rather, these proteins may facilitate the unenergized circulation of sterols among the cell membranes, so that the equilibrium sterol distribution is determined by its relative affinity for the diverse phospholipids in the various organelles.

The level of cholesterol in plasma membranes appears to be limited by the capacity of the high abundance, high affinity phospholipids with which it complexes [15,22]. Modest increments in plasma membrane cholesterol above this physiological set point cause the level of ER cholesterol to increase sharply by about an order of magnitude [9,23]. ER regulatory proteins then respond homeostatically to this signal so as to reduce the sterol excess [9–11,15]. A similar feedback mechanism is seen in mitochondria [14]. In that case, cholesterol that modestly exceeds the cell’s physiological set point stimulates the synthesis of 27-hydroxycholesterol several-fold; this oxysterol then elicits a reduction in the level of cellular cholesterol through several feedback pathways [15]. Cell cholesterol homeostasis could thus be directed by the down-hill transfer of super-threshold cholesterol to regulatory sites in cytoplasmic membranes.

We have now tested a null hypothesis: that the large responses to excess plasma membrane cholesterol observed in the ER and mitochondria reflect a similar increase in the cholesterol in all of the intracellular membrane compartments, driven by its passive equilibration. Our data support this hypothesis: doubling the cholesterol in cultured human fibroblasts does not significantly increase their PM pool size; rather, essentially all of the extra sterol moves to cytoplasmic membranes.

Materials and Methods

Materials

Cholesterol was obtained from Steraloids and phospholipids from Avanti Polar Lipids. HPCD (ave. MW = 1396) and methyl-β-cyclodextrin (ave. MW = 1310) were purchased from Sigma-Aldrich. Cholesterol oxidase (EC 1.1.3.6; Streptomyces sp; reported activity of 43.9 U/mg protein) was obtained from EMD. The BCA protein assay kit was from Pierce.

Cell cholesterol compartmental analysis.

Human fibroblasts were derived from the foreskins of circumcised infants at Rush University Medical Center by a procedure approved in writing by its Institutional Review Board. There are no records of these subjects. The cells were cultured in DME 10 (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum) to ~90%
Analytical procedures

Cholesterol and cholestenone were determined by HPLC [25]. Phospholipid P was determined by phosphomolybdate colorimetry on organic extracts [26,27]. Protein was determined using BCA with BSA as a standard. All assays were carried out at least in duplicate.

Results

Determination of the distribution of cellular cholesterol

Measuring the cholesterol content of individual organelles was not feasible. Instead, we measured the relative size of the pools of unesterified cholesterol in the plasma membrane and the intracellular compartments. We utilized the biphasic kinetics of cholesterol oxidase attack at the cell surface to estimate these two compartments [8]. The method employs glutaraldehyde fixation, to stimulate the oxidation reaction. We infer that the rapidly oxidized compartment is the cell surface cholesterol, while the slower compartment is the intracellular organelles, the cholesterol in which circulates through the cell surface compartment during the incubation with the enzyme. Interpretation of the data also assumes the rapid flip-flop of cholesterol across the plasma membrane and the mobility of the entire sterol pool within the fixed cells [8,28]. The method was validated by an independent technique in a previous study using Niemann Pick C cells that bear large, slowly-circulating endolysosomal cholesterol pools [8].

Applying this approach to normal human skin fibroblasts was more challenging because of the small size of the intracellular cholesterol pool and its more rapid circulation. Nevertheless, the method worked well, as illustrated in Figure 1. While first-order least-squares fits to oxidation time courses were not satisfactory, bi-exponential fits always gave values of $R^2$ of >0.99. The fast (plasma membrane) component was oxidized with half-times of ~1 min in control cells and ~0.5 min in enriched cells, while the slower (intracellular) pool was oxidized as it circulated to the cell surface with a half-time of ~7 min in control cells and ~3.5 min in the enriched cells. Since the cells were fixed and the circulation rapid, we infer that cholesterol was transferred by a non-vesicular pathway [12,19]. On average, control cells contained 35.0 ± 3.9 (SD, n = 10) μg total cholesterol/mg protein. Of this, the PM fraction constituted 76% [i.e., 26.6 ± 5.3 (SD, n = 9)] μg cholesterol/ mg protein; the remaining 24% [i.e., 8.4 ± 4.7 (SD, n = 9)] μg cholesterol/ mg protein was ascribed to intracellular compartments. These results, including the observation that intracellular cholesterol is fully mobile in glutaraldehyde-fixed cells, are in accord with earlier findings [8]. The surprisingly rapid transfer of...
cholesterol among the organelles within fixed cells could be mediated by membrane contact sites that circumvent the diffusion of cytoplasmic carrier proteins [18,19].

Dependence of cholesterol distribution on total cell cholesterol

In various experiments, we increased the cell cholesterol up to two-fold (from ~35 to ~70 μg cholesterol/mg protein) by incubation with HPCD bearing different loads of cholesterol. Incubation for 8 min with a high level of cholesterol enriched the cells about as much as a 3 h incubation with a moderate level of cholesterol, in keeping with other evidence for the rapid equilibration of sterols between β-cyclodextrin donors and PMs at 37°C [28,29]. Increasing the cell cholesterol did not appreciably affect the subsequent growth of the cells or visibly perturb them. (The maximal load of cholesteryl per phospholipid introduced into the PMs here was less than that achieved with red blood cells, also without ill effect [30].) In control experiments (not shown), a doubling of the cell cholesterol was reversed by overnight incubation in growth medium (with a half-time of ~5 h) and by incubation with methyl-β-cyclodextrin for 90 min in DME containing 5% LPDS. The cells grew normally after this reversal.

Surprisingly, the uptake of exogenous cholesterol did not increase the size of the PM pool (Figure 2). That is, the PM of the enriched cells remained essentially unchanged, containing 27.0 ± 4.4 (SD, n = 9) μg cholesterol/mg protein after the various loadings, compared to 26.6 μg cholesterol/mg protein in the control cells. In contrast, the intracellular pool took up virtually all of the cholesterol increment, increasing as much as 5-fold from control cells. In contrast, the intracellular pool took up virtually all of the cholesterol increment, increasing as much as 5-fold from control cells. Exposure to HPCD, since it did not occur in unenriched cells or experimental factors such as the chilling or fixing of the cells or change in the distribution of cell cholesterol was not caused by the kinetics were not analyzed in those experiments [9,10,23].)

Figure 3, the size of the unesterified ER pool was found to increase more than 15-fold during the brief interval that followed the jump in PM cholesterol.

Time course of cholesterol uptake by the ER

To test whether excess cholesterol can move rapidly from the PM to the cell interior in these cells, we followed the response of the ER to a jump in PM cholesterol. The approach involved applying a large pulse of exogenous cholesterol for 6 minutes at 37°C, homogenizing the cells and marking the cholesterol residing in the fragments of ER bearing acyl-CoA:cholesterol acyltransferase by allowing its complete conversion to the radioactive ester form by incubation with [14C]oleoyl-CoA [9]. As shown in Figure 3, the size of the unesterified ER pool was found to increase more than 15-fold during the brief interval that followed the jump in plasma membrane cholesterol. These data support the implication of Figure 2 that increasing cell cholesterol greatly expands the ER pool of excess cholesterol. In that case, all of the cholesterol became completely oxidized with a single rate constant at least as fast as that for the rapid compartment in the intact cells (not shown).

Further evidence on this point comes from a previous study showing that enrichment of PM cholesterol greatly increased its susceptibility to cholesterol oxidase [32]. This makes it implausible that the slow cholesterol pool in enriched cells was a poorly-reactive PM compartment. We also tested whether the large intracellular pool inferred from Figure 2 manifested cholesterol binding by soluble cytoplasmic proteins [12,13,19]. We found, however, that all of the cholesterol detectable in homogenates prepared from both control and enriched cells was pelleted by centrifugation at 100,000 g for 2 h; hence, entirely particulate. We also observed (not shown) that enriched cells labeled with BODIPY-cholesterol had a normal morphology and distribution of membrane fluorescence; in particular, there was no precipitation of the label or conspicuous concentration in particular organelles such as lipid droplets or the endosomal recycling compartment, ERC [13,33].

The disposition of the intracellular cholesterol was further analyzed by centrifuging homogenates to equilibrium on sucrose density gradients; a representative experiment is shown in Figure 4. The cholesterol in both the control and loaded cells was broadly distributed in the gradient with a peak at ~33% sucrose that coincides with the plasma membrane markers previously established for this system [7,34]. Panel A shows that the cholesterol increment in the treated cells was more broadly distributed through the gradient than the control, suggesting that it was not confined to either the plasma membrane or a specific subcellular locus. The homogenate of the enriched cells contained 67% more cholesterol per protein than the control; similarly, the cholesterol recovered from the gradient fractions of the enriched cells was 59% greater than that for the control cells; this indicates that there was no significant loss of cholesterol in the gradient.)

The peak CH/PL mole ratio in the control cells in Figure 4B was 0.74, characteristic of purified plasma membranes [6,7]. The CH/PL mole ratio for the enriched cells was elevated throughout the gradient; while it was only modestly increased in the PM region, it was 2.4-fold higher than the control in the buoyant fractions. These results are in keeping with the hypothesis that the
Figure 4. Gradient distribution of cholesterol in homogenates of control versus cholesterol-enriched fibroblasts. Replicate flasks of fibroblasts were incubated at 37°C for 3.5 h in LPDS in the absence (□) or presence (○) of 1.2 mM HPCD bearing 0.12 mM cholesterol. The control and enriched cells contained 42 and 70 μg cholesterol/mg protein, respectively. The cells were washed, dissociated, homogenized and equivalent amounts of homogenate (measured as protein) spun to equilibrium on sucrose gradients [53]. The gradient fractions were extracted and analyzed for cholesterol (CH) and phospholipid (PL). Panel A, nmol cholesterol/fraction. Panel B, ratios of the mole ratios in enriched versus control in fractions bearing cholesterol/phospholipid, mole/mole, per fraction (left axis) and the phospholipid (PL) concentration (right axis, no symbols). Abscissa, sucrose concentration as weight/volume. A representative experiment. doi:10.1371/journal.pone.0098482.g004

This equilibrium distribution can depend in a complex and unknown fashion on the abundance and characteristics of the various bilayer phospholipids as well as on the amounts of cholesterol present [27]. We therefore defaulted to a simple partition coefficient, $K_3$:

$$K_3 = \frac{([CH_o]/[PL_o])}{([CH_i]/[PL_i])}$$  

Figure 5 presents a simulation with $K_1 = 1,000$, $K_2 = 200$ and $K_3 = 0.1$. (Concentrations are in mole fractions.) The $K_1$ and $K_2$ values as well as the CH/PL stoichiometries employed were in line with literature values for cholesterol-phospholipid interactions [27]. For illustrative purposes, we let CH/PL = 0.37 for the whole unmodified fibroblasts and CH/PL = 0.69 for their PM (These values are marked by the cross in Figure 5). We also assumed that the PM contained 80–90% of the resting cell cholesterol and half of its phospholipid; see Results and refs. [7,8]. In this simulation, PM cholesterol begins to plateau near its physiological resting value just as in Figure 2, although the latter did so much more sharply. In Figure 5, as in Figure 2, the PM retained very little of the excess cholesterol, essentially all of it becoming intracellular. That is, nearly all of the PM cholesterol (○) was in complexes (●) and not free (○). In addition, this simulation shows that there is very little uncomplexed intracellular cholesterol in unmodified cells (i.e., in the region below CH/PL = 0.37) and that surplus cholesterol beyond the physiological set point (cross) goes mostly to the uncomplexed intracellular pool. This prediction is in line with the demonstration of highly thresholded homeostatic responses to super-threshold cholesterol by cytoplasmic effectors—namely, cholesterol esterification in the ER and cholesterol hydroxylation in the mitochondria [9,14,15]. Finally, the CH/PL in the

exogenous cholesterol neither remained in the plasma membranes nor precipitated but, rather, became broadly distributed among the intracellular organelles.

**Modeling the fate of cholesterol in enriched cells.** We used a simple simulation to aid the interpretation of the findings presented in Figure 2. We assumed, as justified below, that cholesterol forms 1:1 complexes with phospholipids in the PM (○) and 1:3 complexes with those in the intracellular membranes (●). Then,

$$CH_o + PL_o \leftrightarrow (CH - PL)_o$$  

$$K_1 = \frac{[CH - PL]_o}{([CH_o] [PL_o])}$$

$$CH_i + 3PL_i \leftrightarrow (CH - PL)_i$$  

$$K_2 = \frac{[CH - PL]_i}{([CH_i] [PL_i]^3)}$$

We also assumed that uncomplexed cholesterol equilibrates between the outer and inner compartments:

$$CH_o \leftrightarrow CH_i$$  

$$K_3 = \frac{([CH_o]/[PL_o])}{([CH_i]/[PL_i])}$$

Figure 5. Simulation of the distribution of complexed and uncomplexed cholesterol in plasma membranes and intracellular membranes as a function of total cell cholesterol. CH/PL denotes moles cholesterol/mole phospholipid. Total plasma membrane cholesterol (○); plasma membrane cholesterol complexes (●); total intracellular cholesterol (▲); uncomplexed intracellular cholesterol (■); complexed intracellular cholesterol (◇); uncomplexed plasma membrane cholesterol (○). The cross designates typical literature values for the CH/PL of unmodified intact fibroblasts and for the CH/PL of their plasma membranes. See the text for a discussion of the values employed. doi:10.1371/journal.pone.0098482.g005
simulated intracellular compartment (▲) ultimately approached that in the PM (●), as in Figure 2.

For the simulation to be concordant with the data, we had to assign a partition coefficient for the uncomplexed cholesterol (Kc) that strongly favored the cytoplasmic compartment. This would be the case if the “free” cholesterol in excess of the stoichiometric equivalence point was less soluble in the continuum of PM CH−PL complexes than in the cytoplasmic membranes. The literature suggests that the PM phospholipids would be significantly more avid for cholesterol than those in the intracellular membranes below the stoichiometric equivalence point for their complexation \( i.e., Kc \gg Ks \) [27]. The unexpected implication of Figures 2 and 5 is that the opposite relationship holds for the partition of the uncomplexed excess sterol \( (i.e., Kc < Ks) \). This strong prediction requires testing; however, we currently lack detailed information concerning the abundance of the multitude of phospholipid species in the various organelles as well as their sterol stoichiometries and affinities both below and above their equivalence points. If the affinity of the cytoplasmic membranes for uncomplexed cholesterol is found not to be significantly higher than that of the plasma membrane, a different explanation must be sought for the results presented herein and in studies of the effect of cell cholesterol levels on its regulation [15].

Discussion

This study was prompted by evidence that the cholesterol pools in ER and mitochondria increase by about an order of magnitude when cell cholesterol is raised modestly [9,15,23]. Figure 2 affirms the null hypothesis that the bulk of cholesterol in excess of the physiological set point of the PM moves to the intracellular organelles at the expense of the cell surface compartment. That is, essentially all of the extra cholesterol load moved quantitatively from the PM to intracellular compartments, increasing their sterol content several-fold. (Some of the excess cholesterol must remain in the PM, however, since small increments greatly increase the susceptibility of the cell surface to cholesterol oxidase attack and to β-cyclodextrin extraction [32].)

Figure 3 shows that the transfer of PM cholesterol to the ER occurs on a time scale of a few minutes. Similar rapid kinetics for the decrease of ER cholesterol were observed when cholesterol was extracted from intact cells with HPCD [9]. These findings are consistent with a variety of other studies suggesting a brisk circulation of cholesterol among cell membranes [9,13,14,15]. The extra intracellular cholesterol was not dissolved in the cytosol or located in precipitates or in a single locus, but was distributed widely, particularly in buoyant membranes (Fig. 4). It is unlikely that the excess cholesterol was brought into the cell through a vesicular pathway, since the rate of uptake of cholesterol from 6 or 8-min pulses was orders of magnitude greater than endocytosis would predict [35]. Rather, the added cholesterol might move down its chemical activity gradient between the PM and the cell interior by any of several transport pathways [13,16–21].

The state of the surfeit of cholesterol in the cytoplasmic membranes can be viewed in two ways. One posits a phase separation or crystallization of excess sterol molecules [2,36,37]. Consistent with this premise, the unsaturated phospholipids enriched in the cytoplasmic membranes are known to have a low solubility limit for cholesterol compared to the saturated phosphocholine species characteristic of PMs [37,38]. This mechanism might also account for the sharp increase in cholesterol accessibility to probes such as cholesterol oxidase and β-cyclodextrins that parallels the solubility limits reported for various phospholipid species [27,37–40]. In fact, cholesterol free of association with phospholipids shows enhanced accessibility to cholesterol oxidase and β-cyclodextrin, at least in condensed monolayer films [41,42].

Arguing against the crystallization hypothesis is the report that more than a day was required for crystals to form from large loads of intracellular cholesterol [43]. It also seems unlikely that cholesterol crystals would serve as a homeostatic signal to regulatory proteins or be a substrate for homeostatic enzymes in the ER and mitochondria. Rather, crystallization should dissipate the elevated chemical potential that appears to drive the association of cholesterol with and consequent stimulation of these proteins; see, for example, refs. [9,11]. Furthermore, two recent molecular dynamics simulations found that excess cholesterol was not crystalline (albeit, using a brief time window) but rather dispersed in the bilayer with a high chemical activity [40,44].

An alternative view is that cholesterol is not simply an indifferent bilayer solute but rather forms (presumably weak and dynamic) complexes of varied stoichiometries and affinities with different membrane phospholipids. While such complexes have not been demonstrated directly, there is considerable evidence for specific chemical associations between sterols and phospholipids, as illustrated in ref. [45]. Cholesterol exceeding stoichiometric equivalence with the phospholipids would remain dissolved and dispersed in the bilayer of complexes in a metastable state [15,22,27,46]. Being less constrained by association with the phospholipids, super-threshold cholesterol molecules would have an elevated chemical activity [40,44]. This would promote their transfer to sites with a lower free energy; in this case, driving the sterol from over-loaded PMs to under-saturated intracellular compartments.

Supporting this hypothesis is diverse evidence for a sharp increase in the accessibility or activity of cholesterol exceeding a characteristic mole ratio with various bilayer phospholipids [15]. Thresholds are seen in diverse contexts: the susceptibility to cholesterol oxidase of cholesterol in PMs and/or phospholipid vesicles; its extraction by β-cyclodextrin; its binding to perfringolysin O toxin; its 27-hydroxylation in mitochondria; its rise in abundance in the ER and the consequent rise in the activation of ER proteins that it regulates [10,11,22,23,27,47,48]. In addition, numerous intercalating amphipaths appear to compete stoichiometrically with and displace cholesterol from phospholipids, providing independent evidence for phospholipid complexation and the enhanced activity and/or accessibility of uncomplexed cholesterol [49,50].

The two aforementioned hypotheses might actually depict different features of the same process. Consider that cholesterol in excess of the capacity of the phospholipids would remain soluble and dispersed in the bilayers of those complexes. The highest level of exogenous cholesterol introduced in these experiments would have led to an overall cellular CH/PL of ≤0.8 (a cholesterol mole fraction of ≤0.44) in both the plasma membranes and cytoplasmic organelles—not an unprecedented bilayer load.] The high chemical activity of the super-threshold fraction could drive its redistribution to other membranes and ligands. In the absence of such alternative opportunities, excess sterol would eventually reach the limit of its solubility in the bilayer and dissipate its high chemical activity through crystallization.

Our data support the view that increments in cell cholesterol will preferentially associate with the high capacity, high-affinity phospholipids in the PM at the expense of the phospholipids in the cytoplasmic membranes with their lower affinity and capacity. Cholesterol rising beyond the stoichiometric equivalence point of the PM phospholipids would be allocated wholesale to intracel-
hilar membranes as in Figures 2 and 5. The consequent increment in ER and mitochondrial cholesterol would then associate with the regulatory proteins therein, eliciting homeostatic responses that limit further cholesterol accumulation [15]. Excess cellular cholesterol is toxic to cells, perhaps because of the formation of crystals and/or through ER stress [51,52]. By holding cholesterol at or below the saturation point for the phospholipids in the cell surface and intracellular bilayers, this feedback system can serve to protect cells from injury.

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Author Contributions

Conceived and designed the experiments: YL, TLS. Performed the experiments: YL, JY. Analyzed the data: YL, TLS. Contributed reagents/materials/analysis tools: YL. Wrote the paper: YL, TLS.

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