Intracellular Trafficking of Cholesterol Monitored with a Cyclodextrin*

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The sterol binding agent 2-hydroxypropyl-β-cyclodextrin is shown to be a convenient and useful experimental tool to probe intracellular pathways of cholesterol. Biochemical and cytochemical studies reveal that cyclodextrin specifically removes plasma membrane cholesterol. Depletion of plasma membrane sphingomyelin greatly accelerated cyclodextrin-mediated cholesterol removal. Cholesterol arriving at the plasma membrane from lysosomes and the endoplasmic reticulum was also removed by cyclodextrin. Cellular cholesterol esterification linked to the mobilization of cholesterol from lysosomes was strongly attenuated by cyclodextrin, suggesting that the major portion of endocytosed cholesterol is delivered from lysosomes to the endoplasmic reticulum via the plasma membrane. Evidence for translocation of lysosomal cholesterol to the endoplasmic reticulum by a plasma membrane-independent pathway is provided by the finding that cyclodextrin loses its ability to suppress esterification when plasma membrane sphingomyelin is depleted. The Golgi apparatus appears to play an active role in directing the relocation of lysosomal cholesterol to the plasma membrane since brefeldin A also abolished cyclodextrin-mediated suppression of cholesterol esterification. Using cyclodextrin we further show that attenuated esterification of lysosomal cholesterol in Niemann-Pick C cells reflects defective translocation of cholesterol to the plasma membrane that may be linked to abnormal Golgi trafficking.

Cholesterol is an integral membrane component required for normal cellular function (1). In mammalian cells this sterol is derived either from low density lipoprotein (LDL) following receptor-mediated endocytosis and subsequent hydrolysis in lysosomes (2) or via de novo biosynthesis in the endoplasmic reticulum (ER) (3). The mechanism for the ensuing movement of cholesterol from these intracellular sites to their ultimate cellular destinations is an unresolved question of fundamental importance to cell biology and medicine. Defects in these transport pathways can alter cellular cholesterol metabolism resulting in pathological states. Niemann-Pick C (NP-C) disease is characterized by sequestration of LDL-derived cholesterol in lysosomes that is linked to delays in the induction of cholesterol- mediated homeostatic responses (4, 5). Development of atherosclerotic lesions involves in part a failure to maintain adequate transfer of cholesterol from intracellular sites of accumulation to the cell surface for removal by extracellular acceptors (6). Understanding pathways of intracellular cholesterol transport is a critical step toward the correction of cellular cholesterol lipidoses.

The intracellular distribution of cholesterol generated by these transport pathways is not uniform, with a major fraction present in the plasma membrane (PM) (7). It has been suggested that this high concentration of cholesterol at the cell surface results in part from its close association with sphingomyelin (SM) (8). Enzymatic digestion of SM by exogenously added sphingomyelinase (SMase) mobilizes PM cholesterol for esterification in the ER (9–12).

Several methods have been employed to monitor intracellular movement of cholesterol. Arrival of cholesterol at the ER is accompanied by esterification catalyzed by the resident enzyme acetyl-CoA:cholesterol acyltransferase. Transfer of cholesterol to the PM has been studied by subcellular fractionation (13), chemical modification by exogenously added cholesterol oxidase (14), or via removal from the cell surface to extracellular cholesterol acceptors (15, 16). This latter approach has been limited by the lack of an acceptor that efficiently removes cholesterol. The effectiveness of cholesterol depletion from the PM by HDL varies with cell type and is neither an extensive nor a rapid process (16, 17).

In the present study we demonstrate the utility of 2-hydroxypropyl-β-cyclodextrin (CD), as an effective extracellular cholesterol acceptor, to monitor the flux of cholesterol through the PM of living cells. Cyclodextrins are cyclic oligomers of glucose that have the capacity to sequester lipophiles in their hydrophobic core (18). The water-soluble cyclodextrins preferentially form inclusion complexes with sterols thereby greatly enhancing their solubility in aqueous solution (19–21). It has recently been shown that CD is capable of stimulating cholesterol efflux from cultured cells with very high efficiency (22). We now report our findings on the use of CD to follow movement of de novo synthesized cholesterol to and from the PM and the egress of LDL-derived cholesterol from lysosomes. We further used CD to reveal that cell surface SM and the Golgi apparatus regulate intracellular transport of cholesterol.

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§ The abbreviations used are: LDL, low density lipoprotein; BFA, brefeldin A; CD, 2-hydroxypropyl-β-cyclodextrin; ER, endoplasmic reticulum; HDL, high density lipoprotein; HM, heavy membrane fraction; LM, light membrane fraction; LPDS, lipoprotein-deficient serum; NP-C, Niemann-Pick type C; PM, plasma membrane; SM, sphingomyelin; SMase, neutral sphingomyelinase; PBS, phosphate-buffered saline.

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**EXPERIMENTAL PROCEDURES**

Materials—Fetal bovine serum was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS) and human fibroblast density lipoprotein (LDL) were prepared by Advanced Biotechnology Laboratories, Rockville, MD. Glass and plastic microscope culture wells (Lab-Tek) were purchased from Thomas Scientific. 9,10-unsaturated fatty acids, 2 mM glutamine, and 100 units of penicillin/streptomycin/ml were prepared by LabServe Inc., Bayonne, NJ. Neutroph sphingomyelinase (from Staphylococcus aureus) was purchased from Sigma. Brefeldin A was purchased from Epicentre Technologies (Madison, WI) and stored as a 1 mg/ml stock solution in ethanol at -20°C.

**RESULTS**

CD Removes Cellular Cholesterol from the Plasma Membrane—2-Hydroxypropyl-β-cyclodextrin (CD) is an effective sterol acceptor that removes the unesterified cholesterol mass from viable cells in a saturable, concentration-dependent manner (Table I). Approximately 70% of cellular cholesterol can be removed after 4 h treatment with 2% CD at 37°C. The kinetics of cholesterol removal from both normal and NP-C fibroblasts are similar with a biphasic nature (Fig. 1). In cells uniformly labeled with [3H]acetate, approximately 40–50% of the [3H]sterol is depleted within 60 min followed by a slower rate of depletion (t1/2 = 5–6 h). It appears that esterified cholesterol mass is expressed as nmol per mg cell protein of six wells (mean ± S.D.). Values in parentheses are expressed as % of initial cell sterol remaining.

**TABLE I**

| Concentration CD | Free* | Esterified* |
|------------------|-------|-------------|
| g/100 ml         |       |             |
| 0                | 81 ± 4 (100) | 16 ± 4 (100) |
| 0.05             | 43 ± 1 (13)  | 7 ± 1 (44)   |
| 1                | 38 ± 2 (47)  | 10 ± 1 (62)  |
| 2                | 24 ± 1 (30)  | 8 ± 2 (50)   |
| 4                | 23 ± 1 (28)  | 10 ± 1 (62)  |
| 8                | 18 ± 1 (22)  | 12 ± 3 (75)  |

* Sterol mass is expressed as nmol per mg cell protein of six wells (mean ± S.D.). Values in parentheses are expressed as % of initial cell sterol remaining.

**Fig. 1. Kinetics of efflux of de novo synthesized [3H]sterol from normal and NP-C fibroblasts to CD.** Subconfluent normal and NP-C human fibroblast cultures were incubated in McCoy's 5% LPDS medium at 37°C for 4 days. Cell monolayers were then incubated with the same medium containing 8.9 μM [3H]acetate (specific activity = 1000 dpm/μmol) at 37°C for 24 h to label PM pools of cholesterol. A portion of the cultures were washed in PBS, and then lipids and protein were extracted in the following solvents as described. The remaining cultures were incubated at 37°C in McCoy's 5% LPDS medium containing 2% CD for the times indicated, washed, and then extracted. Levels of cell-associated [3H]sterol were determined by thin layer chromatography as described. Total levels of cell-associated [3H]sterol formed from [3H]acetate remained constant in the absence of CD. Values are mean ± S.D. of six wells.

**Cytochemical Analyses**—Cells were seeded at a density of 2.4 × 10⁶ cells/well in 6-well plates and incubated with McCoy's medium 5% LPDS for 4 days, washed, and then incubated with the same medium with LDL (50 μg/ml) for 48 h. After washing, cells were incubated with the indicated concentration of CD in McCoy's medium 5% LPDS, 25 μM HEPES, pH 7.2, for 4 h. Total cellular cholesterol and protein were extracted, and cholesterol mass and protein were determined as described under "Experimental Procedures."
cannot be mobilized to an appreciable extent by CD (Table I). The small amount of cholesterol ester removed does not appear to depend on the concentration of CD.

Exposure of normal and NP-C cultures to neutral sphingomyelinase (SMase) for 1 h resulted in the digestion of 80% of \([^{3}H]\)sphingomyelin formed by de novo biosynthesis (data not shown). SMase treatment dramatically enhances both the rate and extent of removal of cellular \([^{3}H]\)sterol by CD (Fig. 2). Treatment with CD following SM digestion resulted in the removal of cellular sterol in a biphasic manner with an initial rapid rate of depletion (70% within 15 min) followed by a subsequent slower rate of depletion \((t_{1/2} = 5-6\) h). Similar results were obtained when cells were treated with CD and SMase concurrently (data not shown). There was no detectable release of lactic acid dehydrogenase to the medium nor altered triglyceride synthesis for up to 2 h of this combined treatment (data not shown).

We employed fluorescence microscopy using filipin, a fluorescent marker specific for unesterified cholesterol (30), to demonstrate that CD primarily targets the PM for removal of cellular cholesterol. In normal cells, we have confirmed (31) that filipin-cholesterol fluorescence is associated with both the PM and intracellular structures (Fig. 3A). Treatment of these cells with CD alone greatly diminishes PM fluorescence with out substantially affecting intracellular staining (Fig. 3B). Filipin-cholesterol fluorescence associated with the PM is essentially eliminated after treatment with CD/SMase (Fig. 3C). In NP-C fibroblasts cultured with LDL, we also confirmed (31) that considerable filipin-cholesterol fluorescence is seen at the PM as well as intense fluorescence in perinuclear lysosomes resulting from defective intracellular mobilization of LDL-derived cholesterol in these cells (Fig. 4A). Treatment of these NP-C cells with CD/SMase resulted in complete loss of filipin-cholesterol fluorescence from the PM with no substantial loss of fluorescence from intracellular sites (Fig. 4B).

CD preferentially removes LDL-derived cholesterol from PM as determined by subcellular fractionation. Following incubation of both normal and NP-C fibroblast cultures with LDL \([^{3}H]\)cholesteryl linoleate for 24 h, a portion of the cultures was subsequently treated with CD/SMase. Subcellular fractionation of cell extracts on 10% Percoll gradients reveals two peaks of \([^{3}H]\)cholesterol representing heavy density (fractions...
1–5) and light density (fractions 10–15) membranes (Fig. 5). A lysosomal marker (β-hexosaminidase) was found predominantly in the heavy membrane fraction (HM), whereas markers for the PM, Golgi, and ER were found in the light membrane fraction (LM) (data not shown). In normal cells, 60–70% of the total recovered radioactivity was distributed in the LM while 18% was associated with the HM (Fig. 5A). Treatment of these cells with CD/SMase reduced the level of [3H]cholesterol in LM and HM by 64 and 50%, respectively. The loss of radioactivity from LM is consistent with the removal of [3H]cholesterol from the PM. [3H]Cholesterol remaining in LM after CD/SMase treatment may in part represent [3H]cholesterol associated with the Golgi complex and/or ER. The loss of [3H]cholesterol from HM is consistent with a partial transfer of [3H]cholesterol from lysosomes to the PM during depletion with CD. In NP-C cells, the subcellular distribution of LDL-derived [3H]cholesterol was higher in HM (30%) and lower in LM (58%) when compared with normal cells (Fig. 5B), reflecting the defective intracellular distribution of lysosomal cholesterol (15). Unlike normal fibroblasts, CD/SMase treatment did not substantially alter the LDL-derived [3H]cholesterol distributed in HM of NP-C fibroblasts. This treatment did, however, produce a decrease by 2/3 in the percentage of the total [3H]cholesterol distributed in LM. Although less lysosomal cholesterol appears at the PM of NP-C cells, a comparable proportion of LDL-derived cholesterol was removed by CD/SMase from this membrane in both cell types.

CD can also prevent internalization of PM cholesterol to intracellular compartments. Fibroblasts were treated with SMase to induce movement of PM cholesterol to the ER (10). This incubation, performed in the presence of [3H]oleate to follow arrival of cholesterol at the ER by esterification, was carried out in the absence or presence of CD. Treatment of fibroblasts with SMase stimulated cholesterol esterification (Fig. 6). This SMase-stimulated esterification of cholesterol is

![Fig. 4. Cytochemical assessment of the effect of CD on the distribution of cholesterol in membranes of NP-C fibroblasts cultured with LDL. NP-C human fibroblasts were incubated in McCoy's/5% LPDS medium at 37°C for 4 days. Cells were then incubated in fresh medium containing LDL (50 μg/ml) for 24 h. A portion of the cultures were washed in PBS and then incubated in McCoy's/5% LPDS medium alone for 1 h at 37°C (A). The remaining cultures were incubated in McCoy's/5% LPDS medium containing 2% CD and 0.12 units/ml SMase for 1 h at 37°C (B). The PM of NP-C fibroblasts demonstrates considerable filipin fluorescence (A, arrowheads). Note that CD/SMase treatment eliminated filipin-cholesterol fluorescence at the PM (B, arrowheads) but did not appreciably alter perinuclear lysosomal filipin-cholesterol fluorescence. All photographic exposures and processing were performed identically. (A and B, × 150).](http://www.jbc.org/)

**Fig. 5.** Effect of combined treatment with CD and SMase on the distribution of [3H]cholesterol derived from LDL-[3H]cholesteryl linoleate in Percoll fractions of normal and NP-C fibroblasts. Stock cultures of normal and NP-C fibroblasts were harvested, and 1.0–1.5 × 10⁶ cells were seeded in 75-cm² flasks in McCoy's/5% LPDS medium at 37°C for 4 days. Cultures were then incubated with fresh medium containing 15 μg/ml LDL[3H]cholesteryl linoleate (specific activity = 3.94 × 10⁷ dpm/mg protein) for 24 h, washed, and then incubated in McCoy's/5% LPDS medium in the absence or presence of 2% CD and 0.12 units/ml SMase at 37°C for 1 h. Cultures were washed and then fractionated in Percoll gradients, as described. 0.5-ml aliquots of Percoll fractions were added to scintillant mixture, and [3H] radioactivity was counted. Total radioactivity associated with NP-C fibroblasts was normalized to the level observed in normal fibroblasts.
markedly suppressed by CD.

CD can be used to monitor arrival at the plasma membrane of cholesterol from intracellular compartments—fibroblasts were incubated with [3H]mevalonate in the presence or absence of CD. A major fraction (80%) of the newly synthesized [3H]sterol was transferred to the medium at 24 h in the presence of CD (Fig. 7B). The relatively small proportion of newly synthesized [3H]sterol (20%) that remained associated with cells may represent intracellular pools or a more highly resistant PM sterol pool.

The arrival of lysosomal cholesterol at the PM can be indirectly monitored by assessing the effect of CD on the level of cholesterol esterified during its release from lysosomes. LDL-derived lysosomal cholesterol has been shown to be transported to the PM where it enriches a precursor pool of cholesterol destined to be esterified in the ER. One would predict that CD can retard cellular cholesterol esterification by removing substrate from the PM. This was tested by loading the lysosomes of cultured fibroblasts with LDL-derived cholesterol using progesterone. During a subsequent period of progesterone washout, cells were incubated with [3H]oleate to assay esterification. Levels of cholesteryl [3H]oleate formed in vitro were determined in lipid extracts of cells by thin layer chromatography as described. Values are mean ± S.D. of triplicate wells.
of lysosomal cholesterol to the PM in the mutant cells. The residual esterification in NP-C cells may represent intracellular routing of lysosomal cholesterol to the ER via a pathway that bypasses the PM.

Plasma Membrane Sphingomyelin Alters the Intracellular Trafficking of Lysosomal Cholesterol—Removal of PM sphingomyelin can affect the intracellular transport path of lysosomal cholesterol. Our experimental protocol was to load the lysosomes of cultured normal fibroblasts with LDL-cholesterol using progesterone. These cells were then briefly treated with SMase to deplete PM SM, followed by washing the progesterone from the cultures to establish cholesterol transport out of lysosomes. During progesterone washout cells were incubated with \([3H]\)oleate in the absence or presence of CD. As established above (Fig. 9), CD alone can effectively suppress esterification of cholesterol in untreated cells (Fig. 10A). Depletion of PM SM alone does not alter the level of esterification of lysosomal cholesterol (cf. Fig. 10, A and B). However, when cells were exposed to SMase, CD no longer blocked cholesterol esterification (Fig. 10B). This loss in the sensitivity of esterification to CD modulation occurs under conditions that render cells particularly sensitive to removal of PM sterol (Fig. 2). These data suggest that depletion of PM SM diverts the trafficking of lysosomal cholesterol away from the PM without diminishing its accessibility to the ER.

BFA Alters the Intracellular Trafficking of Lysosomal and Plasma Membrane Cholesterol—The current studies with CD provide evidence that the Golgi plays a key role in the movement of LDL-derived cholesterol from lysosomes to the PM. Lysosomes of normal and NP-C fibroblasts were loaded with LDL-cholesterol using progesterone. Lysosomal cholesterol transport was re-established with progesterone washout, and cells were incubated with \([3H]\)oleate in the presence or absence of BFA and/or CD. The effective suppression of cholesterol esterification by CD during progesterone washout in normal fibroblasts (Fig. 11A) was largely lost when BFA was added (Fig. 11B). This finding does not result from a loss in the ability of CD to remove \([3H]\)sterol from the PM of BFA-treated cells labeled to equilibrium with \([3H]\)acetate (data not shown). The loss of CD-mediated suppression of esterification in the presence of BFA suggests that disruption of the Golgi complex (39) reduces the intracellular trafficking of lysosomal cholesterol to the PM without substantially diminishing its accessibility to the ER. In NP-C cells, CD does not suppress the attenuated cholesterol esterification either in the presence (Fig. 11C) or absence (Fig. 11D) of BFA. In these mutant cells, BFA stimu-
dishes. Levels of cholesteryl [3H]oleate formed subsequently washed, and lipids and protein were extracted in the presence of 2% CD at 37°C for the times indicated (Fig. 12). In NP-C cells, stimulation of esterification by cotreatment with BFA and SMase is 50% of that seen in normal cells. It appears that BFA-mediated perturbations of the Golgi complex enhance access of SMase-mobilized PM sterol to the ER for esterification.

DISCUSSION

We have demonstrated that CD provides a simple and effective method for monitoring the flow of cellular cholesterol pools to and from the plasma membrane of living cells. Using this approach we have followed the movement of cholesterol derived from LDL or de novo biosynthesis. We have found that the bulk of LDL-derived cholesterol destined for esterification in the ER is first delivered to the PM. In addition we provide evidence that transport of cholesterol from lysosomes via this pathway can be dramatically affected by the integrity of the Golgi complex and the SM content of the PM.

Our experimental approach depends on the effective removal of unesterified cholesterol by CD from viable cells. CD depletes up to 90% of cellular cholesterol from intact, viable cells in a concentration (Table I) and time (Fig. 1)-dependent manner. The kinetics of sterol efflux from human fibroblasts to CD are similar to those recently reported for mouse L-cells (22). The efflux process likely involves cholesterol desorption from cells followed by diffusion to an extracellular acceptor (6, 43). An excess pool of CD, routinely used in our present studies, serves as a sink for cellular cholesterol. Biochemical and cytochemical analyses strongly suggest that CD specifically targets removal of cholesterol from the cell surface. Depletion of cellular cholesterol with CD corresponds to a marked loss of filipin-cholesterol fluorescence from the PM but not from intracellular pools. Subcellular fractionation of normal and Niemann-Pick C cells reveals that CD preferentially removes LDL-derived [3H]cholesterol primarily from light membranes (PM). The kinetics and extent of cholesterol removal from the PM of normal or NP-C fibroblasts by CD are indistinguishable (Fig. 1). This suggests that the defective intracellular transport of cholesterol in NP-C cells (4, 15) is not an intrinsic property of the PM.

The large fraction of cellular cholesterol associated with the PM has often been attributed to its interaction with SM at this site (38). This hypothesis is based on part on observations that cells treated with SMase display a burst of esterification at the ER of cholesterol derived from the PM (9–11). These studies suggested that a large portion of PM cholesterol relocated to intracellular sites that appeared to be inaccessible to extracellular lipid acceptors such as HDL₃ (40, 44). In the present study we find, however, that removal of cellular cholesterol by CD is, in fact, significantly enhanced after enzymatic depletion of PM SM (Fig. 2). Our data are consistent with the recent report that the majority of cellular cholesterol remains at the PM after SM depletion (45). The observed level of enhancement of CD-mediated cholesterol removal after SM depletion suggests that a considerable portion of PM cholesterol (~50%) may be associated with SM. We also show that CD, in contrast to HDL (40, 44), can divert PM cholesterol from intracellular transport pathways, such as movement to the ER for esterification (Fig. 6). This difference most likely reflects the relative effectiveness of CD as a cholesterol acceptor (22).

Because of its effective removal of surface cholesterol, CD provides a facile means for monitoring sterol flux through the PM. CD removes a significant fraction of de novo cellular sterol (Fig. 7). It has been estimated that the half-time for delivery of cholesterol from its site of synthesis in the ER to the cell indicates cholesterol esterification 4-fold (Fig. 11D). The BFA-induced increase in cholesterol esterification is consistent with previous observations (40, 41, 42) and suggests that Golgi cholesterol can be absorbed into the ER along with other Golgi components.

The Golgi also appears to play a role in the movement of PM cholesterol to the ER in normal and NP-C cells. Such movement can be induced by treating cells with SMase and assessed by measuring esterification (10). When cells are treated with either BFA or SMase alone there is a relatively weak stimulation of esterification of PM sterol (Fig. 12). However, when BFA and SMase are both present, there is a dramatic increase in the esterification of PM sterol in normal and mutant cell cultures (Fig. 12). In NP-C cells, stimulation of esterification by cotreatment with BFA and SMase is 50% of that seen in normal cells. It appears that BFA-mediated perturbations of the Golgi complex enhance access of SMase-mobilized PM sterol to the ER for esterification.
surface is 10–30 min in several cell types (46–48). The half-times reported for fibroblasts appear to depend on the methods employed, ranging from 10 min (13) to 1–2 h (49). The reason for these discrepancies remains unclear. Our data (Fig. 7B) show a marked increase in the rate of removal of de novo synthesized sterol to CD after a few hours of incubation with the [3H]mevalonate precursor, consistent with the extended time of transfer in fibroblasts (49). Further studies will enable us to provide a more precise measurement for the rate of transfer.

Transport of LDL-derived cholesterol from lysosomes to the PM can also be followed with CD. We have previously shown that progesterone treatment of normal cells produces an accumulation of LDL-derived cholesterol in lysosomes, as seen with the NP-C mutation (32). After progesterone removal, this cholesterol pool leaves lysosomes. The reversibility of the progesterone-induced block provides a direct method to study egress of cholesterol from lysosomes. We find that removal of LDL-derived cholesterol by CD during progesterone washout occurs with half-times of 4 h in normal cells and 8 h in NP-C cells (Fig. 8). This difference appears to reflect the lesion in lysosomal cholesterol trafficking to the PM reported in the mutant cells (15), since the rates of removal of resident PM sterol itself by CD are identical in the two cell types. Our measurements of removal of lysosomal cholesterol from cells by CD during progesterone washout are considerably longer than those previously reported for the movement of pulse-labeled lysosomal cholesterol to the PM with values of 1–2 min (33), 40–50 min (34), and 1–2 h (15). Differences among these data may be attributable to variability in the cell type and the methods employed. Our measurements may also reflect extensive loading of lysosomes with cholesterol that occurs when cultures are exposed to progesterone during LDL uptake (32). The slower rates of clearance that we report may therefore reflect saturation of the transport pathways that mediate movement of lysosomal cholesterol to the PM as well as the rate at which CD can remove cholesterol from the PM. Nonetheless, it is important to note that we detect a slower rate in the movement of LDL-derived cholesterol to the PM in NP-C cells compared with normal cells.

Movement of cholesterol from lysosomes during progesterone washout can also be monitored through its subsequent esterification (32). This pathway appears to involve an initial transfer of lysosomal cholesterol to the PM since PM cholesterol is considered to provide the principal source of substrate for acetyl-coenzyme A:cholesterol acyltransferase located in the ER (35–38). It has, however, been speculated that a portion of LDL-derived cholesterol destined for esterification may be directly transferred from lysosomes to the ER (36). We find that CD in normal cells can block esterification by as much as 70%.
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Fig. 12. Effect of BFA on SMase-stimulated esterification of PM sterol. Subconfluent normal and NP-C human fibroblast cultures were incubated in McCoy’s/5% LPDS medium at 37 °C for 4 days. Cell monolayers were then incubated with the same medium containing 8.9 μM [3H]acetate (specific activity = 1000 dpm/μmol) at 37 °C for 24 h to label PM pools of cholesterol. Cultures were washed in PBS and then chased in McCoy’s/5% LPDS medium for 24 h at 37 °C. A portion of the cultures was washed in PBS and then incubated in McCoy’s/5% LPDS medium alone or in McCoy’s/5% LPDS medium containing 2 μg/ml BFA for 6 h at 37 °C. The remaining cultures were incubated in McCoy’s/5% LPDS medium containing 0.12 units/ml SMase in the absence or presence of 2 μg/ml BFA for 6 h at 37 °C. Cell monolayers were subsequently washed and then lipids and proteins were extracted in the dishes. Levels of cell-associated [3H]sterol and [3H]ester formed in vitro were determined in lipid extracts by thin layer chromatography as described. Values are mean ± S.D. of six wells.

(Fig. 9) and conclude that this fraction of endocytosed cholesterol, mobilized from lysosomes, is translocated to the ER through a PM-mediated pathway. The remaining sterol fraction presumably moves to the ER by a PM-independent pathway. In NP-C fibroblasts, the attenuated cholesterol esterification associated with the defective lysosomal cholesterol transport pathway is insensitive to suppression by CD (Fig. 9) and thus appears to bypass the PM.

The current studies show that cell surface SM affects the trafficking of lysosomal cholesterol to the PM. In SMase-treated cells, CD no longer suppresses the esterification of lysosomal cholesterol during progesterone washout (Fig. 10). Thus, removal of SM from the PM appears to alter, without apparently attenuating, intracellular trafficking of lysosomal cholesterol. Since esterification of LDL-derived cholesterol in SM-depleted cells is no longer affected by CD, we conclude that it has been re-routed to the ER by a PM-independent pathway. It is conceivable that SM depletion renders the PM incapable of accepting cholesterol delivered from lysosomes. Further investigation is required to evaluate this unexpected finding.

Several studies have provided a link between the Golgi complex and the processing of cellular cholesterol. It has been postulated that the distribution of cholesterol within the complex, increasing in an anterograde cis to trans direction (50), plays a role in the sorting of membrane proteins (51, 53) and lipids (52). We have previously showed that the processing of LDL-derived cholesterol in normal cells is accompanied by an enrichment of the cholesterol content of the Golgi complex in an anterograde fashion (31, 53, 54). By contrast LDL processing in NP-C fibroblasts leads to a premature (31) and abnormal (53) enrichment of cholesterol in Golgi cisternaG. These studies were the first to implicate the Golgi with the intracellular trafficking of LDL-derived cholesterol.

We have now obtained further evidence for a role of the Golgi complex in the transport of LDL-derived cholesterol. Treatment of normal cells with BFA renders esterification of lysosomal cholesterol insensitive to exogenous CD (Fig. 11). This finding implies that cholesterol is normally transferred from lysosomes to the PM via the Golgi and that BFA disruption of this organelle results in re-routing of cholesterol to the ER via a PM-independent pathway. Our present studies also provide further insights to suggest that defective transport of lysosomal cholesterol in NP-C cells may be linked to disruption of its passage through the Golgi. As shown above, in these mutant cells, lysosomal cholesterol appears to be translocated to the ER via a PM-independent pathway, which is both attenuated and insensitive to exogenous CD. A curtailment in cholesterol transfer from lysosomes to the PM in NP-C cells due to a defect in movement through the Golgi complex is suggested by the finding that BFA curtails this transfer in normal cells. The observed 4-fold stimulation of cholesterol esterification in NP-C cells during disruption of the Golgi with BFA is consistent with the notion that abnormal cholesterol accumulation in the Golgi complex leads to an increase in the supply of substrate for acetyl-CoA:cholesterol acyltransferase when Golgi components fuse with the ER.

The Golgi complex also appears to play a role in shuttling cholesterol between the PM and other organelles. Transfer of cholesterol between the Golgi and PM is thought to be involved in the formation of cholesterol-rich PM caveolae (55). The cedared-specific cholesterol binding protein, caveolin (56), cycles constitutively between the PM and intracellular sites including the Golgi complex (57). Our present studies support a retrograde movement of cholesterol from the PM to the ER that may be modulated by the Golgi complex. Mobilization of cholesterol from the PM following SMase treatment results in an enrichment of the cholesterol content in the Golgi complex (data not shown), as monitored by the stabilized fluorescence of C6-NBD-ceramide (54). The movement of cholesterol between the PM and the Golgi may in turn be linked to its appearance in the ER. This is supported by our observation that BFA-induced disruption of the Golgi complex greatly enhances esterification of PM cholesterol mobilized by SM depletion (Fig. 12). This marked enhancement of cholesterol esterification by BFA suggests a direct role for the Golgi in regulating trafficking of PM-derived cholesterol to the ER. Mobilization of PM cholesterol to the ER remains deficient in NP-C cells, even with BFA treatment, suggesting an inherent defect in the processing of cholesterol by the Golgi in these mutant cells. Golgi processing of cholesterol derived from LDL and the PM may play an essential role in maintaining the appropriate distribution of cholesterol within the cell.

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