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Niemann-Pick type C1 protein influences the delivery of cholesterol to the SREBP:SCAP complex

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The proposed role of Niemann-Pick type C1 protein (NPC1) in the delivery of low-density lipoprotein (LDL) cholesterol to the sterol regulatory element binding protein (SREBP):SREBP cleavage activation protein (SCAP) complex in the endoplasmic reticulum has been largely based on indirect studies and remains contentious. The major aim of the present study was to assess whether NPC1 is involved in the delivery of LDL cholesterol to the SREBP:SCAP complex. A cell line stably expressing green fluorescence protein-SCAP was cultured in the presence of U18666A, which can induce a Niemann-Pick type C disease phenotype, in order to locate the SREBP:SCAP complex by fluorescence microscopy. Our major finding was that defective NPC1 caused a delay in the ability of LDL cholesterol to suppress SREBP processing. This was shown in a time-course experiment by the effect of LDL on green fluorescence protein-SCAP movement when cells were treated with pharmacological agents to induce a Niemann-Pick type C disease phenotype. We demonstrated directly by fluorescence microscopy that defective NPC1 causes a delay in LDL cholesterol delivery to the endoplasmic reticulum where SCAP senses cholesterol.

Key words: Niemann-Pick type C1 protein; Low-density lipoprotein cholesterol; Fluorescence microscopy; Sterol regulatory element binding protein complex; Cholesterol homeostasis

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INTRODUCTION

Cells have developed a way to tightly regulate the pathways of synthesis and uptake to maintain cholesterol in the range required for cellular function. The components of the cholesterol homeostatic machinery are located in the endoplasmic reticulum (1). These include sterol regulatory element binding protein (SREBP) binding with SREBP cleavage activation protein (SCAP), which is able to up-regulate cholesterol synthesis and uptake (2,3), and acyl-CoA: cholesterol acyltransferase (ACAT) which is activated by nonesterified cholesterol to catalyze cholesterol esterification.

Niemann-Pick type C (NPC) disease is a rare, recessive, autosomal disorder characterized by the accumulation of unesterified cholesterol in vesicles (4) that have properties of both late endosomes and lysosomes (5). The ability of Niemann-Pick type C1 protein (NPC1) to bind cholesterol has been well addressed (6). In cells from NPC-diseased patients, receptor-mediated endocytosis and hydrolysis of low-density lipoprotein (LDL) appear to be normal. However, there seems to be a problem with delivery of cholesterol to the plasma membrane and cell organelles (7-10). In addition, endogenously synthesized cholesterol may also be sequestered in lysosomes when NPC1 is deficient (11,12).

A defining characteristic of NPC disease is the limited ability of LDL to induce cholesterol esterification by ACAT (13). This implies that NPC1 plays an important role in the delivery of LDL cholesterol to ACAT in the endoplasmic reticulum. Since the SREBP:SCAP complex is also lo-

cated in the endoplasmic reticulum and may be regulated by the same homeostatic pool, it has been proposed that the activity of ACAT is a reflection of SREBP processing (14-16). Thus, for NPC1 disease it has been assumed that a lower amount of cholesterol esterification implies high processing of SREBP, leading to abnormalities in cholesterol homeostasis (17). This suggests a role for NPC1 in delivering LDL cholesterol to the SREBP:SCAP complex. However, the assumption that ACAT activity reflects transport of cholesterol to the endoplasmic reticulum, and hence represents activation of the SREBP pathway was challenged by Du et al. (18). Their study showed that cholesterol delivery to ACAT and to the SREBP:SCAP complex may be separate processes. Thus, one can question the validity of reports that infer effects on SREBP processing from effects on cholesterol esterification. Other studies have also questioned the role of NPC1 in delivering LDL cholesterol to the endoplasmic reticulum (19-21). In particular, using an indirect measurement, Lange et al. (21) found that the amount of cholesterol in the endoplasmic reticulum of NPC1 mutant cells was similar to that of wild-type cells, inferring that cholesterol delivery to the endoplasmic reticulum is normal in NPC disease. Similarly, Frolov et al. (20) observed that the severity of the biochemical NPC1 phenotype did not correlate with the extent of cholesterol esterification.

Although several studies have inferred that cholesterol homeostasis is perturbed in NPC-diseased cells, this has been based on indirect studies and remains contentious. In particular, the proposed role of NPC1 in the delivery of LDL cholesterol to the SREBP:SCAP complex in the endoplasmic reticulum has been largely based on evidence that is not directly related to SREBP processing (17-20). Therefore, whether or not NPC1 functions in the delivery of LDL cholesterol to the SREBP:SCAP complex is unknown.

The major aim of the present study was to assess whether NPC1 is involved in the delivery of LDL cholesterol to the SREBP:SCAP complex in the endoplasmic reticulum. This is based on the hypothesis that cholesterol transport to the SREBP:SCAP complex and to ACAT may be distinct processes. The experiments carried out in this study addressed this by analyzing the effects of NPC1 in the absence or presence of LDL cholesterol on activation of the SREBP pathway in NPC disease. If NPC1 is involved in LDL cholesterol delivery to the SREBP:SCAP complex, it may be considered to act at a common point in the pathway of cholesterol delivery to both ACAT and SREBP. This would provide more direct evidence confirming the results of previous studies which inferred a link between SREBP activation and ACAT activity in NPC disease. However, if LDL cholesterol delivery to the SREBP:SCAP complex is found to be normal in the presence of defective NPC1 cells, this would further confirm the results of Du et al. (18) showing that ACAT esterification does not necessarily indicate the amount of cholesterol in the homeostatic pool as sensed by SCAP. It would also provide evidence that there are different pathways by which cholesterol moves to ACAT and the SREBP:SCAP complex in the endoplasmic reticulum. The novel aspect of the present study is that we employed more direct measures, such as fluorescence microscopy, of SREBP processing than previously utilized by others.

MATERIAL AND METHODS

Material

Compactin, filipin, glycine, U18666A (Figure 1), androstenediol and LDL were from Sigma (St. Louis, MO, USA). L-glutamine, newborn bovine serum (NBS), penicillin-streptomycin, and Trisol reagent were purchased from Invitrogen (Carlsbad, CA, USA). HEPES was provided by Acros Organics (Geel, Belgium). LDL (50 µg/mL) was added at various concentrations in phosphate-buffered solution (PBS). Androstenediol (0.32 µmol/L; Figure 1), compactin (5 µmol/L), mevalonate (50 µmol/L), and U18666A (0.15 µg/mL) were added in ethanol. The final ethanol concentration was kept constant between conditions in each experiment and did not exceed 0.25% (v/v). LDL was prepared as described in Ref. 22. Human LDL, prepared by ultracentrifugation as described in Ref. 23, was kindly provided by the Central Laboratory, Shandong Provincial Hospital. It was desalted using a PD-10 column (Amersham Biosciences, Buckinghamshire, UK) and eluted using 1X PBS. LDL was filter sterilized using a 0.45-µM filter (Millipore, Carrigtwohill, Ireland) and the final concentration was determined using a protein assay kit (Pierce, Rockford, IL, USA). All solvents were analytical reagent grade. CHO/pGFP-SCAP cells (24), a cell line stably expressing green fluorescence protein (GFP)-SCAP, were kindly donated by Drs. Brown and Goldstein (UT Southwestern, Dallas).

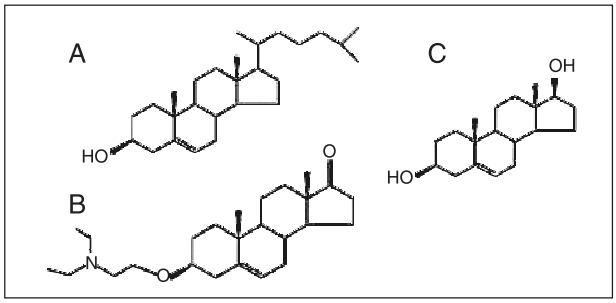

Figure 1. Structure of cholesterol and test pharmacological agents. *A*, Cholesterol. *B*, U18666A. *C*, Androstenediol.

Cell culture

Cells were grown as monolayers at 37°C in the presence of 5% CO_2 in various media as listed.

Media

Medium A, 5% NBS: 1:1 (v/v) Dulbecco's modified Eagle's medium:Ham's F12 containing penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and 5% (v/v) NBS. Medium B, 5% LPDS: 1:1 (v/v) Dulbecco's modified Eagle's medium:Ham's F12 containing penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and 5% (v/v) lipoprotein-deficient NBS. Medium C, medium B plus 5 µM compactin and 50 µM mevalonate.

Filipin staining of cholesterol

Filipin staining can be used to determine the location of cholesterol in the cell because it is a fluorescent polyene antibiotic which binds to cholesterol (2,25).

On day 0, CHO K1 cells (Shanghai Institute of Cells, Chinese Academy of Sciences) were plated at a density of 1.2 x 10^5 cells/mL onto 2 mL medium A in a 6-well plate containing sterile 19 x 19-mm coverslips. After 30 h, test treatments were added in medium C. After a 16-h incubation period, cells were washed three times with filipin staining buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4, and 2 g/L glucose). Cells were fixed with 3% formaldehyde (v/v)/PBS, pH 7.4, for 40 min, then washed twice with PBS. Glycine (50 mM) was added and incubated for 10 min. Filipin (50 µg/mL) was added, the plates were covered with foil and left for 2 h at room temperature. Cells were rinsed twice with PBS and then mounted on glass slides with mounting medium (90% glycerol, 10%/Tris-Cl, pH 8). Images were obtained using an Olympus BX60 Upright Microscope with a SPOT Digital camera using a UV filter set (340-380 nm excitation, 400 nm dichroic and 430 nm long-pass filters).

Fluorescence microscopy

SCAP is an escort protein that carries SREBP from the endoplasmic reticulum to the Golgi apparatus in cholesterol-depleted environments, where specific proteases release the transcription factor (26,27). Thus, in the absence of cholesterol, SCAP is localized in the Golgi. In a situation where there is ample cholesterol, the SREBP:SCAP complex will remain tethered in the endoplasmic reticulum. A stable cell line expressing GFP-SCAP was treated with U18666A, an amphiphile that has been widely used to phenocopy NPC disease and does not affect the binding of cholesterol to NPC1 (28), to determine whether defective NPC1 would affect the localization of SCAP when treated with LDL, and hence, to indicate SREBP:SCAP movement from the endoplasmic reticulum to the Golgi.

On day 0, CHO/pGFP-SCAP cells were added to 6-well plates containing sterile 19 x 19-mm coverslips at 1 x 10^5 cells/mL in 2 mL medium B. On day 2, cells were incubated in medium C with various additions for 6, 9, 12, and 24 h as indicated. After incubation, the cells were fixed with 3% (v/v) formaldehyde for 20 min at 37°C. Cells were rinsed twice with PBS and then mounted on glass slides with mounting medium (90% glycerol and 10% Tris-Cl). Images were obtained using a Leica TCS SP Laser Scanning Spectral Confocal Microscope (Wetzlar, Germany). GFP was excited with the 488-nm laser lines from an argon laser. Confocal image stacks were edited using the Leica confocal software, LCS Lite.

RESULTS

Treatment with U18666A causes accumulation of LDL cholesterol in cells

Filipin staining of cellular cholesterol was used to determine if the U18666A was causing LDL cholesterol to accumulate in cell lysosomes (Figure 2). In the absence of LDL and U18666A, cholesterol was located mainly in the plasma membrane (Figure 2A). In the presence of LDL, cholesterol was located in the plasma membrane and juxtanuclear, indicating Golgi membrane (arrow in Figure 2B). U18666A treatment without LDL addition caused a minor accumulation of cholesterol in what we assumed to be lysosomes (Figure 2C). When cells were cultured with both U18666A and LDL, there was bright staining in small punctate clusters indicative of cholesterol being sequestered in cell lysosomes (Figure 2D). There was also less staining of cholesterol on the plasma membrane, indicating a problem in delivery of cholesterol to the plasma membrane when the cells were cultured with U18666A.

Filipin staining results indicated that U18666A is indeed creating the NPC-diseased phenotype.

Movement of GFP-SCAP in the NPC1 drug-induced phenotype

We analyzed the sterol-sensing ability in the presence of the NPC1-diseased phenotype using direct immunofluorescence of GFP-SCAP. CHO/pGFP-SCAP cells were treated with LDL (Figure 3). In the absence of LDL, cells have a juxtanuclear staining of GFP-SCAP (Figure 3A). This indicates that SCAP is localized in the Golgi, as would be expected in cholesterol-starved cells. Treatment with LDL mostly yielded a more diffuse reticular staining (Figure 3B), indicating that the SREBP:SCAP complex is retained in the endoplasmic reticulum and that the movement to the Golgi apparatus is inhibited.

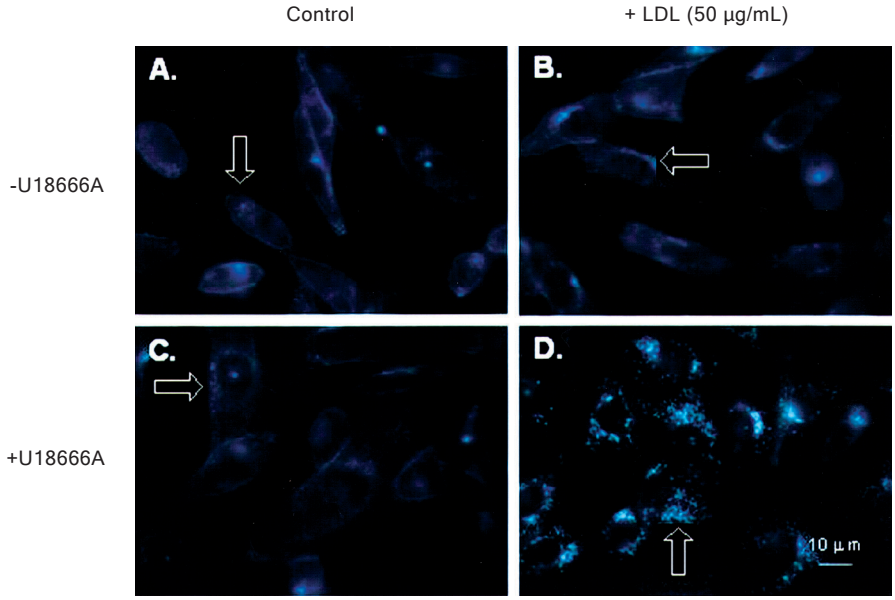

Figure 2. LDL cholesterol accumulates in lysosomes of cells treated with U18666A. CHO K1 cells were seeded on coverslips in medium A as described in the Methods section. CHO K1 cells were incubated in medium C in the absence and presence of LDL (50 µg/mL) and U18666A (0.15 µg/mL) as indicated, for 16 h. Cells were fixed with 3% (v/v) formaldehyde and then stained with filipin (50 µg/mL) for 2 h. Images were obtained using a UV filter. *A*, In the absence of LDL and U18666A, cholesterol was located mainly in the plasma membrane. *B*, In the presence of LDL, cholesterol was located in the plasma membrane and juxtanuclear indicating Golgi membrane. *C*, U18666A treatment without LDL addition caused a minor accumulation of cholesterol lysosomes. *D*, When cells were cultured with both U18666A and LDL, there was bright staining indicative of cholesterol being sequestered in cell lysosomes. Arrows indicate location of cholesterol.

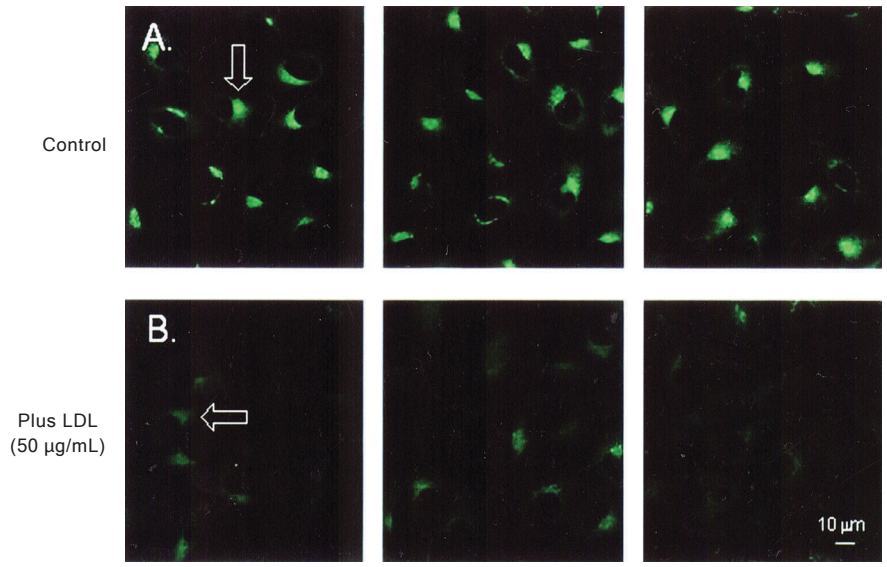

Figure 3. LDL cholesterol causes diffuse reticular staining of GFP-SCAP. CHO/pGFP-SCAP cells were grown in medium B on coverslips and treated in medium C for 9 h in the absence or presence of LDL (50 µg/mL). Cells were fixed with 3% (v/v) formaldehyde and images were obtained using a Lecia TCS SP Laser Scanning Spectral Confocal Microscope. Triplicate fields presented are representative of at least 6 images for N = 2 replicate cultures. *A*, In the absence of LDL, cells have a juxtanuclear staining of GFP-SCAP in the Golgi. *B*, Treatment with LDL, SREBP:SCAP complex is retaining in the endoplasmic reticulum. Arrows indicate location of GFP-SCAP in the Golgi.

In addition, the CHO/pGFP-SCAP cell line was treated with U18666A in the absence or presence of LDL and compared to control (Figure 4). In the absence of LDL, treatment with U18666A causes mostly juxtanuclear staining of GFP-SCAP (Figure 4C) similar to that found in control cells (Figure 4A). However, there also appears to be slightly more reticular staining (Figure 4C). Therefore, due to the similarity of the structure of U18666A to cholesterol (Figure 1A,B), this compound may be having an effect on SCAP cholesterol sensing. In the presence of LDL, U18666A causes SCAP to be more localized in the Golgi apparatus (Figure 4D *vs* B), indicating that U18666A prevents SCAP sensing of LDL cholesterol at the level of the endoplasmic reticulum and therefore continues to transport SREBP to the Golgi apparatus for cleavage.

As a relative control, androstenediol was added to the control condition since it has a structure similar to that of U18666A (Figure 1B,C). Therefore, it may be able to mimic the effect of U18666A on SCAP but is known not to affect NPC1 (29). Experiments were performed using androstenediol to test if it would cause similar localization of GFP-SCAP in the absence of LDL (Figure 5). Furthermore, treatment with androstenediol caused a localization of GFP-SCAP similar to that observed with U18666A in the absence of LDL (Figure 5). It was therefore deemed appropriate that androstenediol should be used as the control in order to account for the slight reticular localization of GFP-SCAP that was caused by U18666A. Using androstenediol as a control, a time-course experiment was performed to determine if there was a delay in SCAP movement when treated with U18666A in combination with LDL. The time points used were 6, 9, 12, and 24 h (Figure 6). In the control condition, GFP-SCAP shifted more to the endoplasmic reticulum staining pattern between 6 to 9 h of LDL choles-

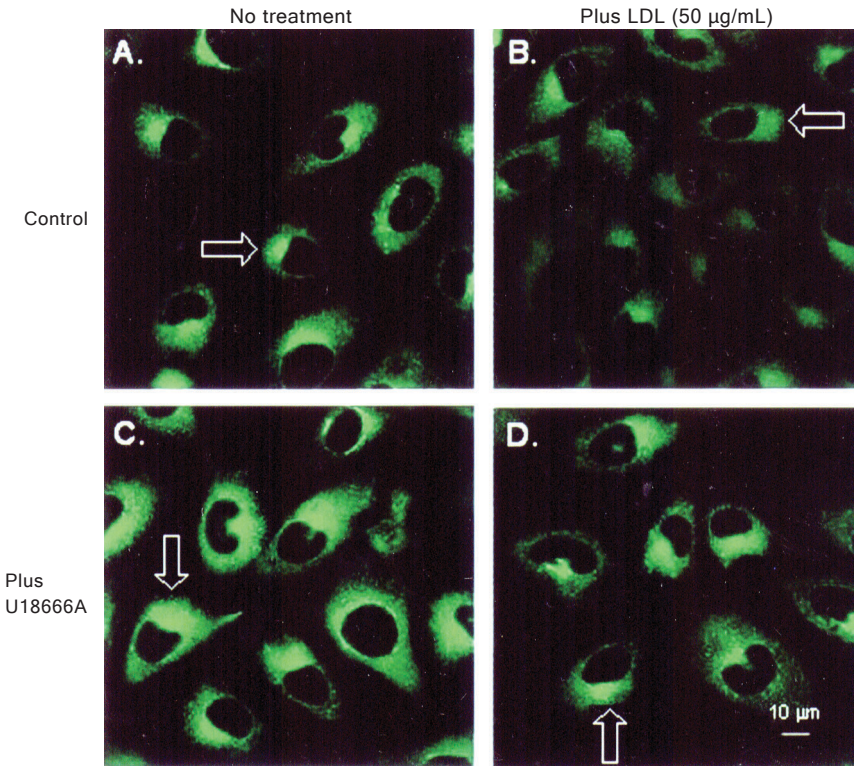

Figure 4. U18666A causes GFP-SCAP movement to the Golgi apparatus in the presence of LDL. CHO/pGFP-SCAP cells were grown in medium B on coverslips and treated with medium C for 6 h in the absence or presence of LDL (50 µg/mL) and in the absence or presence of U18666A (0.15 µg/mL). Cells were fixed with 3% formaldehyde (v/v) and images were obtained using a Lecia TCS SP Laser Scanning Spectral Confocal Microscope. The fields presented are representative of at least 6 images for N = 2 replicate cultures. In the absence of LDL, treatment with U18666A causes mostly juxtanuclear staining of GFP-SCAP (Figure 4C) similar to that found in control cells (Figure 4A). However, there also appears to be slightly more reticular staining (Figure 4C). In the presence of LDL, U18666A causes SCAP to be more localized in the Golgi apparatus (Figure 4D *vs* B), indicating that U18666A prevents SCAP sensing of LDL cholesterol at the level of the endoplasmic reticulum and therefore continues to transport SREBP to the Golgi apparatus for cleavage. Arrows indicate location of GFP-SCAP mostly in the Golgi (A,C,D) and endoplasmic reticulum (B).

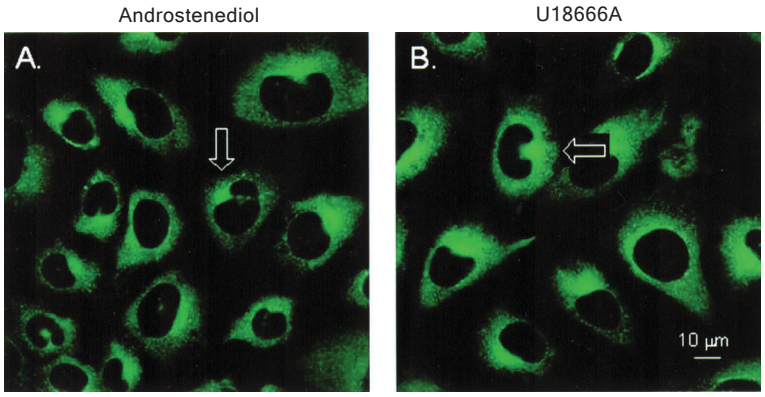

Figure 5. Androstenediol causes a pattern of GFP-SCAP similar to that observed with U18666A in the absence of LDL (arrows). CHO/pGFP-SCAP cells were grown in medium B on coverslips and treated with medium C for 6 h in the presence of either androstenediol (A, 0.32 µM) or U18666A (B, 0.15 µg/mL equivalent to 0.32 µM). Cells were fixed with 3% (v/v) formaldehyde and images were obtained using a Lecia TCS SP Laser Scanning Spectral Confocal Microscope. The fields presented are representative of at least 6 images for N = 2 replicate cultures.

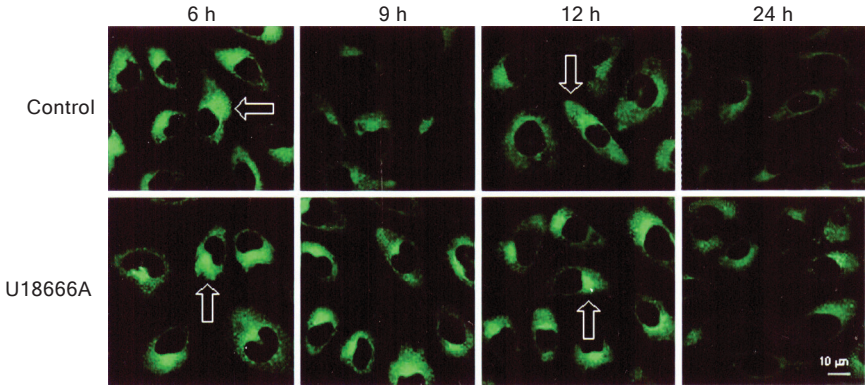

Figure 6. U18666A causes a delay in the ability of LDL cholesterol to suppress SCAP movement to the Golgi apparatus. CHO/pGFP-SCAP cells were grown in medium B on coverslips and treated with medium C for 6, 9, 12, and 24 h in the presence of LDL (50 (µg/mL) and either androstenediol (control, 0.32 µM) or U18666A (0.15 µg/mL equivalent to 0.32 µM). Cells were fixed with 3% formaldehyde (v/v) and images were obtained using a Lecia TCS SP Laser Scanning Spectral Confocal Microscope. The fields presented are representative of at least 6 images for N = 2 replicate cultures. Arrows indicate location of GFP-SCAP.

terol treatment (Figure 6). This is consistent with SCAP sensing LDL cholesterol and being held in the endoplasmic reticulum by the retention protein, Insig-1. Treatment with U18666A maintained Golgi localization of SCAP up to the 12-h time point. However, by 24 h this effect had disappeared (Figure 6), implying that U18666A caused a delay in the ability of SCAP to sense LDL cholesterol.

DISCUSSION

The major finding of the present study was that defective NPC1 caused a delay in the ability of LDL cholesterol to suppress SREBP processing. This was shown in a time-course experiment by the effect of LDL on GFP-SCAP movement when cells were treated with pharmacological agents to induce an NPC-diseased phenotype. This is the first time that a direct immunofluorescence approach was used to demonstrate that defective NPC1 causes a delay in LDL cholesterol delivery to the endoplasmic reticulum where SCAP senses cholesterol.

The cholesterol in the regulatory homeostatic pool is sensed by SCAP in order to determine SREBP processing (26). SCAP travels with SREBP to the Golgi apparatus when there is low cholesterol, and, when there is high cholesterol, the SREBP:SCAP complex is retained in the

endoplasmic reticulum (26). Therefore, the location of SCAP reflects SREBP movement and processing. We treated the cell line that stably expressed GFP-SCAP with U18666A in order to determine activation of the SREBP pathway. We found that U18666A caused a slight retention of SCAP in the endoplasmic reticulum in the absence of LDL. This could be due to its structural similarity to cholesterol. In order to provide a control that would account for the movement of SCAP which occurs when treated with U18666A, we added androstenediol to our control conditions. Androstenediol caused slight retention of GFP-SCAP in the endoplasmic reticulum similar to that observed with U18666A, but did not affect NPC1 (2).

The time-course experiment revealed a delay in delivery of LDL cholesterol to the homeostatic pool in the endoplasmic reticulum as measured by GFP-SCAP. By the 24-h time point it appears that LDL cholesterol has been able to reach SCAP in the endoplasmic reticulum to suppress SREBP:SCAP movement to the Golgi apparatus. These results indicate a role for NPC1 in the delivery of LDL cholesterol to SREBP:SCAP. These results also suggest that, when this NPC1 pathway is inhibited by U18666A, there may be another pathway by which LDL cholesterol can reach SREBP:SCAP. This pathway takes longer for LDL cholesterol to reach the endoplasmic reticulum.

The explanation for the delay in SREBP:SCAP suppression in response to LDL cholesterol in NPC-diseased cells has been linked to a cholesterol “sink” (30,31). This implies that the lysosomes are able to accommodate only a fixed amount of cholesterol. When further LDL cholesterol is added to the lysosomes, the “sink” overflows, spilling cholesterol to the various organelles of the cell including the endoplasmic reticulum, resulting in suppressed SREBP:SCAP movement. Thus, in normal cells NPC1 participates in the delivery of LDL cholesterol from the lysosomes to the membranes, including the endoplasmic reticulum. However, when there is defective NPC1, LDL cholesterol builds up in the lysosomes until they are full. The addition of more LDL cholesterol causes free cholesterol to leak across to other cellular membranes. Our GFP-SCAP results, showing a delayed response to LDL cholesterol, support this theory.

A possible alternative to, or even extension of the “sink” theory is that there may be more than one pathway by which cholesterol moves to the endoplasmic reticulum. While NPC1 participates in the major pathway involved in LDL cholesterol delivery to SREBP:SCAP in the endoplasmic reticulum, there may be a secondary pathway that is independent of NPC1. Thus, in normal cells NPC1 may be involved in regulating the movement of LDL cholesterol to SREBP:SCAP in order to immediately activate the homeostatic responses of the cells, while another, slower pathway, also operates to facilitate cholesterol transport to the endoplasmic reticulum. When NPC1 is defective, the immediate responses of SREBP:SCAP do not occur, but the secondary pathway eventually delivers LDL-derived cholesterol to this complex in the endoplasmic reticulum.

While this image-based study has found that NPC1 is involved in delivering LDL cholesterol to SREBP:SCAP, further study is required to more fully understand this process. Cholesterol esterification and synthesis assays could be useful to address this mechanism and it would also be useful to perform a time-dependent cholesterol esterification assay based on the time points used for the GFP-SCAP experiment. This would determine if LDL cholesterol is eventually able to induce cholesterol esterification by ACAT in NPC1 disease similar to the delay found in LDL cholesterol delivery to SREBP:SCAP. The best way to directly measure SREBP:SCAP processing would be Western blot analysis in cells with NPC1 mutations compared to wild type. Unfortunately, a reliable antibody for SREBP is not commercially available.

In summary, defective NPC1 causes abnormalities in the homeostatic responses to LDL-derived cholesterol in the cell. This study shows that defective NPC1 causes a delay in the delivery of LDL cholesterol to SREBP:SCAP in the endoplasmic reticulum. Therefore, it directly shows that functional NPC1 is required for delivery of LDL cholesterol to SREBP:SCAP.

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