Correction of Apolipoprotein A-I-mediated Lipid Efflux and High Density Lipoprotein Particle Formation in Human Niemann-Pick Type C Disease Fibroblasts*

Emmanuel Badau1, Hong Y. Choi2,3, Diana W. K. Lee3, Emma I. Waddington1, Teddy Chan3, Bela Asztalos5, Jean E. Vance1,4, Alicia Chan3, Graciela Castro3,4, and Gordon A. Francis1,2,3,4

From the Departments of 1Medicine, 2Pediatrics and 3Biochemistry and the Canadian Institutes of Health Research Group in Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta T6G 2S2, Canada, the 4Jean Mayer United States Department of Agriculture Human Nutrition Center on Aging, Tufts University, Boston, Massachusetts 02111, and the 5Institut Pasteur de Lille, UMR545 INSERM, Université de Lille II, 59019 Lille Cedex, France

Impaired cell cholesterol trafficking in Niemann-Pick type C (NPC) disease results in the first known instance of impaired regulation of the ATP-binding cassette transporter A1 (ABCA1), a lipid transporter mediating the rate-limiting step in high density lipoprotein (HDL) formation, as a cause of low plasma HDL-cholesterol in humans. We show here that treatment of human NPC1/−/− fibroblasts with the liver X receptor (LXR) agonist TO-901317 increases ABCA1 expression and activity in human NPC1/−/− fibroblasts, as indicated by near normalization of efflux of radiolabeled phosphatidycholine and a marked increase in efflux of cholesterol mass to apoA-I. LXR agonist treatment prior to and during apoA-I incubation resulted in reduction in filipin staining of unesterified cholesterol on the particle surface by lecithin:cholesterol acyltransferase (LCAT) (7), and transfer to the HDL pool of surface components of triglyceride-rich lipoproteins during their hydrolysis (8). The presence of approximately half-normal HDL-C levels in individuals heterozygous for ABCA1 mutations (9, 10), however, indicates ABCA1 activity is a critical determinant of HDL-C levels in plasma, and that passive efflux and further steps in HDL maturation do not compensate for an initial decrease in ABCA1 activity to increase HDL-C levels.

Niemann-Pick type C (NPC) disease is a neurovisceral disorder characterized by accumulation of unesterified cholesterol and other lipids in late endosomes and lysosomes and impaired cholesterol trafficking to other cell compartments (11, 12). Consistent with impaired regulation of cholesterol synthesis and esterification and low density lipoprotein (LDL) receptor activity in this disorder (13–15), we previously demonstrated that basal and cholesterol-stimulated expression of ABCA1 is diminished in fibroblasts from a patient with NPC disease, leading to impaired lipiddation of apolipoprotein A-I (apoA-I) (16). We also found that 17/21 (81%) of NPC disease patients initially screened had low plasma HDL-C (16). These findings indicate that impaired regulation of ABCA1 leading to low plasma

5 The abbreviations used are: HDL, high density lipoprotein; apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A1; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; DMEM, Dulbecco’s modified Eagle’s medium; FFA, fatty acid-free albumin; LCAT, lecithin:cholesterol acyltransferase; LXR, liver X receptor; LDL, low density lipoprotein; NPC, Niemann-Pick type C; PBS, phosphate-buffered saline; PLTP, phospholipid transfer protein; UC, unesterified cholesterol; TBST, Tris-buffered saline containing 1% Tween 20.
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HDL-C is an integral feature of NPC disease. To our knowledge, NPC disease represents the first known condition of decreased HDL formation and low HDL-C as a consequence of impaired ABCA1 regulation, rather than mutation.

The relationship of impaired ABCA1 regulation and HDL formation to the neurodegeneration and overaccumulation of cholesterol and other lipids in tissues including the liver in NPC disease is currently unknown. Earlier work by Liscum and Faust (15) had shown that addition of the oxysterol 25-hydroxycholesterol could normalize the rate of cholesterol esterification in human NPC disease fibroblasts, despite concomitant suppression of endogenous cholesterol synthesis in these cells. These results suggested the oxysterol might facilitate the mobilization of stored cholesterol from the late endosome/lysosome compartment to the endoplasmic reticulum. Lange and colleagues (17) subsequently showed that addition of oxysterols including 25-hydroxycholesterol to human NPC disease fibroblasts preferentially reduced lysosomal cholesterol and increased endoplasmic reticulum cholesterol, as measured by the pool of cholesterol available for esterification in whole cell homogenates. These investigators also suggested that reduction of endogenous cholesterol synthesis in the presence of oxysterols might deplete plasma membrane cholesterol, resulting in a shift of lysosomal cholesterol to the plasma membrane even in the presence of the NPC1 mutation (17). Frolov et al. (18) reported impaired oxysterol generation in human NPC1 and NPC2 disease fibroblasts, and decreased accumulation of total cholesterol mass and filipin staining of late endosomes/lysosomes in human NPC disease cells incubated with LDL-containing serum in the presence of 25- or 27-hydroxycholesterol. Together, these results suggest that correction of oxysterol-dependent gene regulation normalizes cholesterol trafficking even in the presence of mutations in the genes encoding NPC1 or NPC2.

A major regulator of ABCA1 expression is oxysterol-dependent activation of the nuclear receptor liver X receptor (LXR), which up-regulates ABCA1 to mobilize excess cell cholesterol by forming HDL (19–21). In the present studies we tested whether addition of the non-oxysterol agonist of LXR, TO-901317, would correct the regulation of ABCA1 and normalize lipid efflux to apoA-I and HDL particle formation in human NPC disease fibroblasts, in the absence of direct effects of exogenous oxysterols on cholesterol synthesis and LDL receptor expression. Our results demonstrate correction of ABCA1 expression and near normalization of ABCA1-mediated lipid efflux, as well as correction of ABCG1 expression and HDL particle formation, even in the presence of NPC1 mutations, with LXR agonist treatment. These results suggest LXR agonists might greatly improve or possibly normalize the trafficking and overaccumulation of cholesterol and other lipids in NPC disease.

EXPERIMENTAL PROCEDURES

Materials—Cholesterol, phosphatidylcholine, LXR agonist TO-901317, fatty acid-free bovine serum albumin (FAPA), and filipin were purchased from Sigma. [1,2-3H]Cholesterol (48 Ci/mmol), [methyl-3H]choline chloride (75 Ci/mmol), and [cholesteryl-1,2,6,7,3H]cholesteryl linoleate (84 Ci/mmol) were purchased from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from BioWhittaker, lipoprotein-deficient serum and fetal bovine serum from HyClone, and 25- and 27-hydroxycholesterol from Steraloids, Inc.

Preparation of Lipoproteins and ApoA-I—HDL (d = 1.07–1.21 g/ml) and LDL (d = 1.019–1.063) were obtained from pooled plasma of healthy volunteers by standard ultracentrifugation techniques (22). The whole protein fraction of HDL was obtained by delipidating HDL, and purified apoA-I was obtained using the method of Yokoyama et al. (23) but substituting Q-Sepharose Fast Flow (GE Healthcare) for DEAE-cellulose. Radiolabeling of LDL using [1,2,6,7-3H]cholesteryl linoleate was performed as described (24), to a specific activity of 16–44 cpm/ng of LDL protein. Plasma for determination of HDL particle species was obtained from a 12-month-old male NPC disease subject homozygous for the I1061T mutation of NPC1 who had hepatosplenomegaly and early neurologic symptoms, and 13-month-old male plus 43-year-old female control subjects following informed consent.

Cell Culture—Normal human fibroblasts (NPC1+/+, CRL-2076) were purchased from the American Type Culture Collection. NPC1 compound heterozygote human fibroblasts containing NPC1 mutations I1061T and P237S (NPC1+/−, GM3123) were purchased from the Human Mutant Cell Repository. NPC1+/− cells were plated at 15,000 cells/16-mm well, 50,000 cells/35-mm dish, or 400,000 cells/100-mm dish, and NPC1−/− cells were plated at 20,000 cells/16-mm well, 70,000 cells/35-mm dish, or 600,000 cells/100-mm dish, and grown to confluence in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Where indicated confluent cells were loaded with DMEM containing 2 mg/ml FAPA plus 30 µg/ml non-lipoprotein cholesterol for 24 h as previously described (16). Cells loaded with unlabeled or labeled LDL cholesterol were grown for the last 40% to confluence in DMEM containing 10% lipoprotein-deficient serum to up-regulate LDL receptor expression prior to cholesterol loading.

Labeling of Cellular Cholesterol Pools and Phospholipids—Cells in 16-mm wells were labeled with LDL-derived cholesterol by incubation with DMEM containing 1 mg/ml FAPA (DMEM/FAPA) plus 50 µg/ml [3H]cholesteryl linoleate-labeled LDL protein for 24 h. Following cholesterol loading, cells were rinsed twice with PBS containing 1 mg/ml bovine serum albumin (PBS/bovine serum albumin) at 37°C, and incubated an additional 24 h to allow hydrolysis of added LDL and equilibration of LDL-derived cholesterol in DMEM/FAPA, in the absence or presence of 5 µM TO-901317 added from a 10 mM stock in dimethyl sulfoxide (Me2SO). Cells were rinsed 3 times with PBS/bovine serum albumin prior to analysis or addition of efflux medium. To label phosphatidylcholine, cells in 35-mm dishes were loaded with non-lipoprotein cholesterol for 24 h and then incubated with 5 µCi/ml [3H]choline chloride in DMEM/FAPA ± TO-901317 during the 24-h equilibration period (16). Cells were rinsed 5 times with PBS/FAPA prior to addition of efflux medium.

Cholesterol and Phospholipid Efflux—Following radiolabeling and equilibration steps, cells were incubated for 24 h in DMEM/FAPA containing 10 µg/ml apoA-I in the absence or
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presence of 5 μM TO-901317. After incubation, efflux media were collected and cells were rinsed twice with ice-cold PBS/bovine serum albumin and twice with ice-cold PBS. Cells were stored at −20 °C until lipid extraction. Efflux media were centrifuged at 3,000 × g for 10 min at 4 °C to remove cell debris. Radioactivity in medium was measured directly (for cells labeled with [3H]cholesterol) or the medium was extracted for determination of radiolabeled phosphatidylcholine (25). Extracted cellular lipids were separated by thin-layer chromatography and assayed for radioactivity as previously described (26). Protein content of extracted cell layers was determined using bovine serum albumin as standard (27).

**Cholesterol Mass Assay**—Cells grown in 60-mm dishes, loaded with 50 μg/ml unlabeled LDL, and equilibrated for 24 h in the absence or presence of 5 μM TO-901317 were incubated with 10 μg/ml apoA-I ± TO-901317 for 24 h. After incubation, media and cells were collected and cells were homogenized by sonication. Phospholipids from media or cell homogenates were digested by phospholipase C to remove the polar head groups, and total lipids were extracted in the presence of tridecane as the internal standard. Samples were derivatized with Sylon BFT (Supelco) and analyzed by gas chromatography (Agilent Technologies, 6890 Series equipped with a Zebron capillary column (ZB-5, 15 m × 0.32 mm × 0.25 μm) and connected to a flame ionization detector; Zebron, Palo Alto, CA). The oven temperature was raised from 170 to 290 °C at 20 °C/min, and then to 340 °C at 10 °C/min where the temperature was kept for 24 min. Helium was used as the carrier gas. The gas chromatography was operated in constant flow mode with a flow rate of 4.5 ml of helium/min. The injector was operated in the split mode and was kept at 325 °C, and the detector was kept at 350 °C (28). Separation of sterols was identified by comparing their retention times with standards, and calculation of sterol mass in samples was based on the internal standard.

**Filipin Staining**—Cells were grown on coverslips and loaded with 50 μg/ml LDL as described above. The cells were then incubated in the absence or presence of 5 μM TO-901317 during the 24-h equilibration period and during a 24- or 48-h incubation with DMEM/FAFA ± 10 μg/ml apoA-I. Cells were then fixed with 3% paraformaldehyde in PBS for 20 min, washed three times with PBS, and stained with filipin as described (18), with slight modification. Cells were incubated in PBS with 1.5 mg/ml glycine for 10 min, washed three times with PBS, and stained with 50 μg/ml filipin in PBS for 30 min. Coverslips were mounted with ProLong Antifade reagent (Molecular Probes), containing particles were detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (Calbiochem) in TBST containing 1% nonfat milk for 1 h at room temperature. ApoA-I-containing particles were detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (Calbiochem) in TBST containing 1% nonfat milk for 1 h at room temperature, and then with 125I-labeled donkey anti-rabbit antibody (Amersham Biosciences). The specific activity of the secondary antibody was 4.8 × 10⁶ cpm/mg. Membranes were incubated for 3 h in 80 ml of TBST containing 1% nonfat milk and 2.6 μg of antibody, followed by three washes of 5 min each in TBST before autoradiography (30).

**Semi-quantitative Reverse Transcription-PCR**—cDNA was generated from total RNA isolated from fibroblasts as previously described (16). Amplification of cDNA was performed by initially denaturing DNA at 95 °C for 3 min followed by 95 °C for 75 s. For ABCG1 amplification, annealing was at 60.9 °C for

**Two-dimensional Gel Electrophoresis of HDL Particles**—Fasting blood from the NPC subject and normallipidemic control subjects was collected into EDTA tubes and placed immediately on ice. Plasma was obtained by centrifugation at 2000 × g for 10 min at 4 °C. To characterize apoA-I-containing particles generated by normal and NPC1 human skin fibroblasts, fibroblast-conditioned media in 35-mm dishes were centrifuged at 2000 × g for 5 min at 4 °C to pellet cells and the supernatant was concentrated 10-fold by ultrafiltration (Amicon Ultra-4, MWCO 100000, Millipore). Plasma and media samples were kept on ice and used the same day or frozen at −80 °C. Plasma HDL particles were separated according to the method of Asztalos et al. (10) as previously described. HDL particles in equivalent volumes of concentrated apoA-I-conditioned media were separated according to the method of Castro and Fielding (29) except that in the second dimension, voltage was increased from 100 V for 19 h to 125 V for 24 h to increase the separation of the α-migrating HDL species. Briefly, 20-μl samples were separated in the first dimension by 0.75% agarose gel in 50 mM barbital buffer, pH 8.6, at 200 V for 5.5 h at 5 °C. Electrophoresis in the second dimension was performed with a 2–23% polyacrylamide concave gradient gel at 125 V for 24 h at 5 °C in 0.025 M Tris, 0.192 mM glycine buffer, pH 8.3. High molecular weight protein standards (7.1–17.0 nm, Amersham Biosciences) were run on each gel. Following electrophoresis, samples were electrotransferred (30 V, 24 h, 4 °C) onto nitrocellulose membranes (Trans-Blot, Bio-Rad). To locate the standard proteins, the nitrocellulose membranes were stained with Ponceau S and the position of each protein marked. Membranes were blocked by a 1-h incubation in Tris-buffered saline containing 1% Tween 20 (TBST) and 10% nonfat milk at room temperature. ApoA-I-containing particles were detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (Calbiochem) in TBST containing 1% nonfat milk for 1 h at room temperature, and then with 125I-labeled donkey anti-rabbit antibody (Amersham Biosciences). The specific activity of the secondary antibody was 4.8 × 10⁶ cpm/mg. Membranes were incubated for 3 h in 80 ml of TBST containing 1% nonfat milk and 2.6 μg of antibody, followed by three washes of 5 min each in TBST before autoradiography (30).
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75 s, and extension at 72 °C for 55 s for a total of 33 cycles, with a final extension period of 5 min. ABCG4 amplification was performed using similar conditions except with a total of 29 cycles. An annealing temperature of 56.2 °C over 28 cycles was used for apoE, LCAT, and phospholipid transfer protein (PLTP) amplification, whereas cholesterol ester transfer protein (CETP) required 36 cycles and an annealing temperature of 58.5 °C. All cycle numbers were within the linear range of the PCR. PCR products were visualized on a 1.1% agarose gel, stained with ethidium bromide, and visualized under UV light. All PCR were performed in tandem with cyclophilin control (16). The primers used were: ABCG1, 5′-CACCTGCACATTGGGATCG (forward), 5′-GCCAGGTAGTGGGCTTCCAG (reverse); ABCG4, 5′-GGTGTCCTCTGAGCAGG (forward), 5′-CTTGGTCTCATGAGGG (reverse); apoE, 5′-CCTGGGTCAGATACGCCTGCAGG (forward), 5′-ACTCC (reverse); ABCG4, 5′-GGTGTCCTCATGAGGG (reverse); and LCAT, 5′-GACCCCTGTGGATGGGTGACCCTTGTGCAGG (forward), 5′-TTGGGATCG (reverse).

agonists on proprotein cholesterol, when compared with confluence in 10% fetal bovine serum or loaded with non-cholesterol NPC1 ligands can correct consistent with impaired oxysterol- and LXR-target gene regulation. JOURNAL OF BIOLOGICAL CHEMISTRY

RESULTS

Diminished ABCA1 Expression in NPC1+/- Human Fibroblasts Is Corrected by LXR Agonists—We previously demonstrated low basal and cholesterol-stimulated levels of ABCA1 mRNA and protein in human NPC1+/- fibroblasts (16), consistent with impaired oxysterol- and LXR-target gene regulation in this disease. To determine whether exogenous LXR ligands can correct ABCA1 expression in NPC disease cells, NPC1+/- fibroblasts were incubated in the presence of the synthetic non-oxysterol LXR agonist TO-901317 (31) or the oxysterols 25- and 27-hydroxycholesterol. As shown previously (16), ABCA1 protein levels were low in NPC1+/- cells grown to confluence in 10% fetal bovine serum or loaded with non-cholesterol cholesterol, when compared with NPC1+/- fibroblasts (Fig. 1). Addition of TO-901317 or either of the oxysterols to non-cholesterol-loaded NPC1+/- cells increased ABCA1 protein to levels similar to those seen in cholesterol-loaded or LXR ligand-treated NPC1+/- cells. Similar changes were seen in ABCA1 mRNA levels assessed by reverse transcriptase-PCR (data not shown), indicating the effect of the agonists on ABCA1 is at the transcriptional rather than post-transcriptional level. Reprobing the same Western blots for loading controls protein-disulfide isomerase and actin showed variable results between cell lines, for reasons that are unclear but likely related to alteration of other pathways in NPC1+/- cells. These results indicate equivalent loading of lanes for each condition within each cell type, however, and do not alter our conclusion that ABCA1 expression is increased by LXR agonists in NPC1+/- cells.

ApoA-I-mediated Efflux of Phosphatidylcholine and LDL-derived Cholesterol Is Increased in NPC1+/- Fibroblasts Treated with LXR Agonist—We previously reported impaired efflux of LDL-derived, whole cell, plasma membrane, and newly synthesized cholesterol as well as the phospholipids phosphatidylcholine and phosophomycelin to apoA-I from human NPC1+/- fibroblasts (16). To determine whether increased ABCA1 expression in response to LXR agonist corrects lipid efflux to apoA-I, we measured the delivery of [3H]phosphatidylcholine derived from [3H]choline and [3H]cholesterol derived from [3H]cholesterol linoleate-labeled LDL to apoA-I from cells incubated in the presence or absence of 5 μM TO-901317. Cells were incubated in the presence of TO-901317 for 24 h prior to determination of ABCA1 protein by Western blotting. The presence of LXR agonist raised expression of ABCA1 protein to levels similar to those seen in cholesterol-loaded and LXR ligand-treated NPC1+/- cells, as measured by increased ABCA1 protein bands relative to those in non-cholesterol loaded NPC1+/- cells. The membrane was reprobed with anti-protein-disulfide isomerase (PDI) and anti-actin. Results are representative of three experiments with similar results.

![FIGURE 1. Correction of ABCA1 expression in NPC1+/- human fibroblasts treated with LXR agonists.](image)

**ApoA-I-mediated Efflux of Phosphatidylcholine and LDL-derived Cholesterol Is Increased in NPC1+/- Fibroblasts Treated with LXR Agonist**—We previously reported impaired efflux of LDL-derived, whole cell, plasma membrane, and newly synthesized cholesterol as well as the phospholipids phosphatidylcholine and sphingomyelin to apoA-I from human NPC1+/- fibroblasts (16). To determine whether increased ABCA1 expression in response to LXR agonist corrects lipid efflux to apoA-I, we measured the delivery of [3H]phosphatidylcholine derived from [3H]choline and [3H]cholesterol derived from [3H]cholesterol linoleate-labeled LDL to apoA-I from cells incubated in the presence or absence of 5 μM TO-901317. Cells were incubated in the presence of TO-901317 for 24 h prior to and during the efflux period to apoA-I. As shown in Fig. 2, ~50% less [3H]phosphatidylcholine and LDL-derived [3H]cholesterol expressed as counts/min/mg of cell protein were removed to apoA-I from NPC1+/- cells when compared with NPC1+/- cells in the absence of LXR agonist. The presence of LXR agonist raised efflux of [3H]phosphatidylcholine from NPC1+/- cells to levels higher than from apoA-I-treated NPC1+/- cells, and to ~90% of levels seen from apoA-I plus LXR agonist-treated NPC1+/- cells (Fig. 2A). This result suggests ABCA1 function is restored to normal or near normal levels in NPC1+/- cells by addition of the LXR agonist, and that ABCA1 can mobilize phosphatidylcholine to apoA-I even in the presence of NPC1 mutations. Addition of LXR agonist to NPC1+/- cells raised the efflux of LDL-derived [3H]cholesterol to apoA-I to levels similar to those in apoA-I-treated NPC1+/- cells, but to only 42% of levels in apoA-I plus LXR agonist-treated NPC1+/- cells (Fig. 2B). Efflux of LDL-derived [3H]cholesterol to apoA-I from NPC1+/- cells would be expected to be lower than from NPC1+/- cells even in the presence of LXR agonist, due to dilution of exogenously derived [3H]cholesterol by the
much larger pool of unlabeled unesterified cholesterol in NPC1−/− compared with NPC1+/+ cells (32).

Apo-A1 plus LXR Agonist Treatment Depresses NPC1−/− Fibroblasts of Cholesterol Mass—To determine total cholesterol efflux from both cell types, we measured changes in cholesterol mass in the medium and cellular compartments of LDL-loaded wild type and NPC disease fibroblasts treated with apo-A1 in the absence or presence of LXR agonist. Total cholesterol mass in the medium of apo-A1-treated NPC1−/− cells was slightly lower than in the medium of apo-A1-treated NPC1+/+ cells (Fig. 3A). Cholesterol mass efflux to apo-A1 from NPC1−/− cells was significantly lower than from NPC1+/+ cells after subtraction of albumin-dependent cholesterol efflux to the medium (Fig. 4), which was higher from NPC1−/− cells.

Consistent with previous reports (13, 32), NPC1−/− fibroblasts loaded with LDL and incubated with albumin alone showed ~50% less cholesteryl ester (CE) mass (Fig. 3B), and 2–3-fold more unesterified cholesterol (UC) mass (Fig. 3C) when compared with NPC1+/+ fibroblasts. Apo-A1 treatment markedly depleted CE mass in NPC1−/− cells (Fig. 3B), further accentuated by addition of LXR agonist, consistent with ABCA1 preferentially mobilizing cholesterol that would otherwise be esterified by acyl-CoA:cholesterol acyltransferase (33). This effect was not seen in NPC1−/− cells treated with apo-A1 alone; addition of LXR agonist in the apo-A1 incubation resulted in significant (33%) depletion of CE mass in NPC1−/− cells. Incubation with 10 μg/ml apo-A1 for 24 h failed to reduce UC mass significantly in either NPC1+/+ or NPC1−/− cells (Fig. 3C). Addition of LXR agonist to the apo-A1 incubation resulted in a 30% drop in UC mass in NPC1+/+ cells, and a 28% drop in NPC1−/− cells (Figs. 3C and 4). These results suggest correction of ABCA1 expression also restores the ability of apo-A1 to deplete NPC1−/− cells of cholesterol mass, even in the presence of NPC1 mutations.

A hallmark of NPC disease cells is accumulation of large amounts of cholesterol in late endosomal/lysosomal compartments, as determined by heavy staining with the unesterified cholesterol-specific dye filipin in the same pattern as LAMP1 or LAMP2 staining for these intracellular compartments (34, 35). Filipin staining of NPC1+/+ and NPC1−/− fibroblasts was performed to assess changes upon incubation with apo-A1 in the absence or presence of LXR agonist. Twenty-four and 48-h incubations of NPC1−/− fibroblasts with apo-A1 resulted in no significant alteration in the intense filipin staining when compared with cells treated with albumin alone (Fig. 5). Addition of LXR agonist during the apo-A1 incubations resulted in decreased filipin staining at 24 h, an effect that was accentuated at 48 h. These results are consistent with the drop in UC mass in these cells (Fig. 3C), and suggest up-regulation of LXR responsive genes in NPC1−/− cells is capable of depleting late endosomal/lysosomal cholesterol in the absence of NPC1 protein function.

HDL Particle Species in NPC Disease Plasma and NPC Disease Fibroblast-conditioned Medium—We previously reported the presence of low plasma HDL-C levels in more than 80% of homozygous NPC patients studied (16). To attempt to correlate this finding with changes in cholesterol mass efflux to apo-A1 from NPC disease fibroblasts, we performed two-dimensional gel electrophoresis of HDL particles in the plasma of an NPC disease patient and in apo-A1-conditioned medium of NPC1−/− cells. HDL in the plasma of a 12-month-old male NPC disease subject showed a near absence of large α-1 and preα-1, and a decrease in α-2 and preα-2 HDL particles compared with HDL spe-
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...cies seen in an age- and sex-matched control subjects and a 43-year-old female control subject (Fig. 6A). These changes in HDL species in NPC disease plasma are similar to those previously reported for Tangier disease heterozygote (ABCA1+/−) subject plasma, which also showed decreased levels of α-1, pre-α-1, α-2, and pre-α-2 HDL particles (Fig. 6B) (10). These results suggest that decreased ABCA1 activity in NPC disease, as in heterozygous Tangier disease, is primarily responsible for the absence of larger α-HDL in NPC patient plasma. The absence of these larger, cholesterol-rich α-HDL, which have a longer half-life in plasma than smaller HDL particles (29, 36), likely explains the diminished plasma HDL-C concentration in NPC disease.

Two-dimensional gel electrophoresis of HDL particles formed in the medium of NPC1−/− cells incubated with 10 μg/ml apoA-I for 24 h similarly showed a near absence of larger α-HDL, and fewer small α-HDL, when compared with apoA-I conditioned medium of NPC1+/+ human fibroblasts (Fig. 7A). Addition of LXR agonist during the apoA-I incubation resulted in a marked increase in α-HDL particle formation in both cell types, and converted the HDL species formed by NPC1−/− cells to a normal pattern (Fig. 7B). These results provide further evidence of correction of HDL particle formation by an LXR agonist in the presence of NPC1 mutations.

Impaired ABCG1 Expression in NPC Disease—In addition to reduced expression of ABCA1, the impaired cholesterol trafficking and oxysterol generation in NPC disease could be expected to reduce the expression of all LXR-dependent genes (37). Whereas ABCA1 is believed to be the primary promoter of the initial lipidation of apoA-I in HDL particle formation (3), further delivery of cholesterol to HDL particles occurs in part through the actions of another ABC transporter, ABCG1, whose expression is also LXR dependent (4, 5, 38). The level of ABCG1 expression in human NPC disease has not previously been reported. Like ABCA1 (16), we found lower basal levels of ABCG1 mRNA in LDL-loaded human NPC1−/− fibroblasts when compared with NPC1+/+ cells (Fig. 8). ABCG1 mRNA rose to a similar level in both cell types upon incubation with LXR agonist. These results suggest the correction of HDL particle formation in LXR agonist-treated NPC1−/− fibroblasts may be mediated in part by increased ABCG1 expression, in addition to increased ABCA1 expression.

Additional LXR response genes expected to show impaired expression in NPC disease and involved in cholesterol efflux and modulation of HDL particle size include ABCG4 (5, 39), apoE (40), CETP (41), and PLTP (42). No ABCG4 mRNA was detected in Me2SO- or TO-901317-treated NPC1+/+ or NPC1−/− human fibroblasts (Fig. 8). ApoE mRNA was not found in either of our fibroblast lines, consistent with a previous report in human fibroblasts (43). CETP mRNA was not also found in our fibroblast lines in the absence or presence of LXR agonist. PLTP is thought to mediate formation of larger HDL particles through particle fusion (44), and is LXR-agonist responsive (42). We were, however, unable to detect diminished PLTP mRNA in NPC1−/− fibroblasts, nor an increase in PLTP expression following incubation with TO-901317 in either NPC1−/− or NPC1+/+ fibroblasts. LCAT mediates the maturation of HDL particles by esterifying cholesterol on the HDL particle surface; LCAT has not been reported to be LXR responsive. Cholesteryl ester levels were very low in the efflux medium from both NPC1+/+ or NPC1−/− fibroblasts (data not shown), further suggesting altered LCAT activity is unlikely to explain the increase in HDL particle size seen in LXR agonist-treated NPC1−/− fibroblasts. We were
unable to detect LCAT mRNA in Me2SO- or TO-9012317-treated NPC1<sup>++/+</sup> or NPC1<sup>−/−</sup> fibroblasts.

**DISCUSSION**

The present studies demonstrate that an exogenous agonist of the nuclear receptor LXR can correct the impaired regulation and activity of ABCA1 in human NPC disease cells. The increased ABCA1 protein in NPC1<sup>−/−</sup> cells is functional, as indicated by near normalization of phosphatidylcholine and markedly increased cholesterol efflux to apoA-I. ABCA1 is believed to preferentially mobilize to the plasma membrane the regulatory substrate pool of intracellular cholesterol, likely
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residing in late endosomes/lysosomes (45), that would otherwise be delivered to the endoplasmic reticulum for esterification by acyl-CoA:cholesterol acyltransferase. Removal of this acyl-CoA:cholesterol acyltransferase substrate pool was suggested by the ability of apoA-I to deplete cholesteryl ester mass in LXR agonist-treated NPC1−/− cells, as seen in NPC1+/+ cells incubated in the absence or presence of LXR agonist. In addition, LXR agonist treatment resulted in a marked decrease in unesterified cholesterol mass, as well as late endosome/lysosome cholesterol assessed by filipin staining, in NPC1−/− cells.

Increased expression and activity of ABCA1 by LXR agonist TO-901317 treatment has been shown in multiple cell lines and tissues (31, 46–48). In the current studies we show the ability of increased expression of ABCA1 by this agonist to markedly increase the mobilization of total and late endosomal/lysosomal cholesterol even in the presence of dysfunctional NPC1 protein. Increased ABCA1 in NPC1−/− fibroblasts could be mobilizing cholesterol mainly from the plasma membrane, with secondary depletion of late endosome/lysosome cholesterol by an NPC1-independent mechanism, to replenish plasma membrane cholesterol lost to efflux. Alternatively, or in addition, ABCA1 might be mobilizing cholesterol from the late endosome/lysosome compartment directly, by a mechanism also not requiring functional NPC1. Several studies have suggested internalization of ABCA1 for direct mobilization of cholesterol from the late endosomal/lysosomal compartment represents a quantitatively important component of total ABCA1-mediated cholesterol efflux to apoA-I (35, 49–51). Regardless of whether ABCA1 is removing cholesterol from the late endosomal/lysosomal compartment directly or indirectly, our results indicate up-regulation of ABCA1 can largely bypass NPC1 mutations and increase cholesterol trafficking and removal from NPC disease cells.

The near normalization of phospholipid efflux to apoA-I from LXR agonist-treated NPC1−/− fibroblasts suggests NPC1 function is not necessary for this aspect of ABCA1 activity, either at the plasma membrane or in intracellular compartments. The failure of LXR agonist treatment to completely correct cholesterol efflux to apoA-I, despite normalization of ABCA1 expression, suggests two possibilities. The first is that incubation of NPC1−/− fibroblasts with LXR agonist and apoA-I requires incubations longer than 24 h to see a more pronounced effect. Increased depletion of UC at 48 h, as indicated by a further drop in filipin staining at this time point (Fig. 5), suggests this might be the case. The second possibility is that increased ABCA1 can largely but not completely correct cholesterol mobilization from late endosomes/lysosomes in the presence of NPC1 mutations, and that NPC1 function is necessary for a portion of cholesterol removal from this compartment independent of ABCA1. Whereas this might also be the case, our results suggest increased expression of ABCA1 alone is able to bypass the majority of the effects of NPC1 mutation on intracellular cholesterol trafficking for efflux to apoA-I, and as such, that NPC1 function is not absolutely essential for this process.

The persistent increase in cholesterol content in NPC1−/− cells even after LXR agonist and apoA-I treatment could be due in part to increased de novo cholesterol synthesis in these cells. Previous studies showed similar decreases in de novo cholesterol synthesis in response to the LXR agonist 25-hydroxycholesterol in NPC1+/+ and NPC1−/− cells (15). This suggests the persistently higher UC levels in NPC1−/− cells is more related to the need for longer incubations to see further declines in this pool, rather than an over-compensation of new cholesterol synthesis by the NPC1−/− cells.

We also found near absence of larger α-1 and reduced α-2 HDL particles in the plasma of a 1-year-old NPC disease patient. Subjects heterozygous for ABCA1 mutations show the same pattern of loss of larger α-HDL species (Fig. 6) (10), and approximately half-normal plasma HDL-C levels (9). These results suggest that the reduction in HDL-C and loss of larger α-HDL in NPC patient plasma are due mainly to reduced ABCA1 expression and activity in this disease. Treatment with LXR agonist corrected the pattern of absence of large α-HDL and reduced smaller α-HDL in NPC1−/− fibroblast apoA-I-conditioned medium to the pattern of HDL species seen in NPC1+/− cell apoA-I-conditioned medium. These results provide further evidence that NPC1 protein dysfunction in NPC1−/− cells can be bypassed to normalize the cholesterol trafficking required for HDL particle formation.

The current studies also demonstrate impaired expression of ABCG1 in human NPC disease cells. ABCG1 is an additional LXR response gene involved in cholesterol efflux. Expression of ABCG1, along with ABCA1 and other LXR response genes including apoE, would be expected to be decreased in cholesterol-replete or cholesterol-loaded tissues in NPC disease, due to the sequestration of unesterified cholesterol in late endosomes/lysosomes, and the consequent defect in generation of LXR-activating oxysterols (18). LXR agonist treatment also corrected ABCG1 expression in human NPC1−/− fibroblasts (Fig. 8). Despite our conclusion that the primary mediator of the correction of cholesterol mobilization and HDL particle formation by LXR agonist in NPC1−/− cells was increased ABCA1 activity, these results suggest ABCG1 may also have played a role in this effect. ABCG1 has been shown to facilitate delivery of cell cholesterol to pre-formed HDL but not to lipid-free apoA-I, by a mechanism that does not involve HDL particle binding to the cell surface (5). Overexpression of ABCG1 redistributes cholesterol to cell surface domains where the cholesterol is accessible to removal by HDL (52). LXR agonist TO-901317 treatment of mouse macrophages results in redistribution of ABCG1 from intracellular compartments to the plasma membrane, with a concomitant increase in cholesterol efflux to HDL (53). The relative importance of increased ABCA1-mediated cholesterol efflux to apoA-I versus ABCG1-mediated cholesterol efflux to preformed HDL in correcting the trafficking and removal of cholesterol from NPC disease cells requires further investigation. The absence of expression of LXR response genes apoE and ABCG4 in human fibroblasts ruled out a role for these genes in the correction of lipid mobilization and HDL formation in our studies, but would not rule out an important role of these genes in the impaired cholesterol homeostasis in other NPC disease tissues, including the brain.

Whether the results of studies in human NPC disease cells can be corroborated using cells from mouse models of NPC disease is not yet clear. Npc1-deficient mice show no decrease...
in plasma HDL-C (54, 55), whereas more than 80% of human NPC patients studied to date do (16). This difference is seen despite the recent report of low ABCA1 expression in Npc1<sup>−/−</sup> mouse fibroblasts (56), as seen in our previous studies of human NPC disease fibroblasts (16) and the current studies. These findings suggest differences in HDL metabolism in mice compared with humans might make the Npc1<sup>−/−</sup> mouse an unsuitable model to study this aspect of NPC disease. Studies using the same LXR agonist as used here have shown increased ABCA1 and ABCG1 mRNA in the brains of wild type mice treated with the agonist orally in vivo (48), and increased ABCA1 and ABCG1 mRNA in wild type mouse glial cells in culture (48, 57), although different effects of the agonist on cholesterol efflux from these cells to apoA-I were reported (48, 57). Although no differences were seen in ABCA1 and ABCG1 mRNA levels in the cerebellum of wild type and Npc1<sup>−/−</sup> mice, the increase in cerebellar ABCA1 and ABCG1 expression with TO-901317 treatment might have contributed to the protection against Purkinje cell death and extended lifespan seen in Npc1<sup>−/−</sup> mice treated with the neurosteroid allopregnanolone (56). Additional studies are needed to know how well expression of ABCA1 and ABCG1 in fibroblasts correlates with expression of these transporters in the brain of human patients with NPC disease, and whether up-regulation of these transporters in the brain might contribute to increased survival in these patients.

In summary, we have demonstrated the ability of an agonist of the nuclear receptor LXR to up-regulate ABCA1 expression and activity and largely correct phospholipid and cholesterol efflux to apoA-I, as well as HDL particle formation, in the presence of NPC1 mutations. In addition, ABCG1 expression was found to be low in NPC1<sup>−/−</sup> cells, and also corrected with LXR agonist treatment. Addition of LXR agonist during apoA-I incubations markedly decreased late endosomal/lysosomal cholesterol staining by filipin, as well as total unesterified cholesterol mass in NPC1<sup>−/−</sup> cells. These results suggest the NPC1 protein is non-essential for the trafficking and removal of cell cholesterol if the downstream defects in ABCA1 and ABCG1 expression induced by dysfunctional NPC1 can be corrected (37). Whether correction of ABCA1 and/or ABCG1 expression would be sufficient to have a major therapeutic benefit in human NPC disease, or whether NPC1 has another essential role in addition to mobilization of cholesterol from late endosomes/lysosomes, are questions of major importance.

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