Niemann-Pick Type C2 protein contributes to the transport of endosomal cholesterol to mitochondria without interacting with NPC1*

Barry E. Kennedy, Mark Charman, and Barbara Karten

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, B3H 4R2, Canada

Abstract  Mitochondrial cholesterol is maintained within a narrow range to regulate steroid and oxysterol synthesis and to ensure mitochondrial function. Mitochondria acquire cholesterol through several pathways from different cellular pools. Here we have characterized mitochondrial import of endosomal cholesterol using Chinese hamster ovary cells expressing a CYP11A1 fusion protein that converts cholesterol to pregnenolone at the mitochondrial inner membrane. RNA interference-mediated depletion of the voltage-dependent anion channel 1 in the mitochondrial outer membrane or of Niemann-Pick Type C2 (NPC2) in the endosome lumen decreased arrival of cholesterol at the mitochondrial inner membrane. Expression of NPC2 mutants unable to transfer cholesterol to NPC1 still restored mitochondrial cholesterol import in NPC2-depleted cells. Transport assays in semi-permeabilized cells showed nonvesicular cholesterol trafficking directly from endosomes to mitochondria that did not require cytosolic transport proteins but that was reduced in the absence of NPC2. Our findings indicate that NPC2 delivers cholesterol to the perimembrane region of late endosomes, where it becomes available for transport to mitochondria without requiring NPC1. —Kennedy, B. E., M. Charman, and B. Karten. Niemann-Pick Type C2 protein contributes to the transport of endosomal cholesterol to mitochondria without interacting with NPC1. J. Lipid Res. 2012. 53: 2632–2642.

Supplementary key words  VDAC1 • MLN64 • semi-permeabilized cells • Chinese hamster ovary cells • NPC1-interaction domain • pregnenolone • F2 protein

Cholesterol influences the biophysical properties of membranes and the function of transmembrane proteins. In mammalian cells, a dynamic network of cholesterol trafficking pathways maintains cholesterol content of each subcellular membrane within a characteristic range to ensure optimal function. Cholesterol moves among membranes by vesicular transport, on soluble sterol carrier proteins, or across sites of membrane apposition (1–3).

Mitochondria are cholesterol-poor organelles, especially in their inner membrane, but cholesterol is nevertheless essential for mitochondrial function. Cholesterol delivery to the mitochondrial inner membrane controls oxysterol and steroid synthesis. Moreover, mitochondrial cholesterol may affect ATP synthesis, glutathione import, and reactive oxygen species generation (4–6). The mechanisms of cholesterol transport to the mitochondrial inner and outer membranes are not fully elucidated. In steroidogenic cells, cholesterol delivery to the mitochondrial inner membrane is mediated by the soluble steroidogenic acute regulatory protein (StAR), which interacts with several proteins in the mitochondrial outer membrane, including the translocator protein (formerly known as peripheral benzodiazepine receptor), several translocator protein binding proteins (7, 8), and the voltage-dependent anion channel (VDAC) 1 (9). Depending on availability and environmental conditions, cholesterol from the plasma membrane (10), endosomes, or endoplasmic reticulum (ER) (11, 12) can supply mitochondria of nonsteroidogenic cells with the low levels of cholesterol needed for oxysterol formation and membrane maintenance.

The transport of endosomal cholesterol to the plasma membrane and ER is crucially dependent on the Niemann-Pick Type C proteins NPC1 and NPC2. Loss of function of either of these proteins causes cholesterol accumulation in late endosomes and defects in cholesterol homeostasis.

Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; F2, fusion protein of CYP11A1-adrenodoxin-adrenodoxin reductase; LAMP1, lysosome associated membrane protein 1; LPDS, lipoprotein-deficient serum; MLN64, metastatic lymph node protein 64; 22-OH Chol, 22R-hydroxycholesterol; NPC, Niemann-Pick Type C; RNAi, RNA interference; SF, serum-free; siRNA, short interfering RNA; StAR, steroidogenic acute regulatory protein; VDAC, voltage-dependent anion channel.

1To whom correspondence should be addressed.

*e-mail: bkarten@dal.ca

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(13), and leads to the fatal, neurodegenerative NPC disease. NPC1 is anchored in the late endosomal perimeter membrane by 13 transmembrane domains, forming three large loops in the endosomal lumen and a sterol-sensing domain with homology to several other proteins of cholesterol metabolism and regulation (14). NPC2 is a small, soluble sterol-binding protein in the endosome lumen, which mediates cholesterol transfer between membranes (15–19). According to a recent model, NPC1 and NPC2 interact to mediate the bulk of cholesterol egress from late endosomes by a mechanism in which NPC2 extracts cholesterol from inner membranes of late endosomes, binds to the second luminal loop of NPC1, and transfers cholesterol to the N-terminal loop domain of NPC1 for distribution throughout the cell (20–22).

Our previous work demonstrated that in Chinese hamster ovary (CHO) cells, endosomal cholesterol reached the mitochondrial inner membrane even in the absence of functional NPC1, whereas depletion of the late endosomal protein MLN64 decreased the arrival of cholesterol at the mitochondrial inner membrane (11). Since MLN64 localizes to the endosomal perimeter membrane and has a cytosolic cholesterol-binding START domain that is homologous to StAR (23), our findings suggested a direct endosome-to-mitochondria cholesterol trafficking pathway that is mediated by MLN64 but bypasses NPC1.

Here we further investigate mitochondrial cholesterol import using CHO cells in which pregnenolone synthesis is used to report cholesterol transport to the mitochondrial inner membrane due to stable expression of a CYP11A1 fusion protein. Depletion of candidate proteins by RNA interference revealed contributions of NPC2 and VDAC1 to mitochondrial cholesterol import. Expression of NPC2 with point mutations in the sterol-binding or in the putative NPC1-interaction domain was used to determine which aspects of NPC2 function were necessary for mitochondrial cholesterol import. Measurement of mitochondrial cholesterol import in semi-permeabilized cells suggested that cholesterol could be directly transferred from endosomes to mitochondria in a nonvesicular pathway involving NPC2, MLN64, and VDAC1, but not NPC1.

MATERIALS AND METHODS

Materials

Cell culture media, FBS, and other media supplements were obtained from Life Technologies (Burlington, ON, Canada). Trilostane and 22R-hydroxycholesterol (22-OH Chol) were purchased from Steraloids (Newport, RI). Unless otherwise indicated, other materials were obtained from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Ottawa, ON, Canada). Human LDL were prepared from plasma of healthy, normolipidemic volunteers by density gradient ultracentrifugation (24) with approval from the Human Research Ethics Board, Dalhousie University (protocol #2007-1493). Lipoprotein-deficient serum (LPDS) was prepared by density ultracentrifugation from FBS (24).

Cell lines

Monoclonal lines of CHO and NPC1-deficient CHO (4-4-19) cells stably transfected with pcDNA3.1-F2 were described previously (11). pcDNA3.1-F2 encodes a fusion protein of human CYP11A1 (P450 side chain cleavage complex), adrenodoxin reductase, and adrenodoxin, which is targeted to the mitochondrial inner membrane where it converts cholesterol to pregnenolone (25, 26). pcDNA3.1-F2 was generated previously from an pExe-F2 expression vector, which was a gift from Dr. Walter Miller (University of California at San Francisco, San Francisco, CA). NPC1-deficient 4-4-19 cells expressing a nonfunctional NPC1 protein with a point mutation (G660R) in the sterol-sensing domain (27, 28) were provided by Dr. Laura Liscum (Tufts University, Boston, MA). Cells were maintained in Ham’s F12 medium with 5% FBS and 300 µg/ml geneticin. CHO cells stably expressing cytosolic mCherry protein were generated by transfection with pmCherry-C1 (Clontech) and selection with 300 µg/ml geneticin.

Expression vectors

V81A, V81D, and Y119S mutations (leading to point mutations in V62 and Y100 in mature NPC2) were introduced into human NPC2 (pYFP-NPC2 vector obtained from Dr. Heiko Runz (University of Heidelberg, Heidelberg, Germany) by an overlap-extension PCR procedure using mutagenesis and flanking primers listed in the supplementary data (29)). Mutant cDNAs (without YFP tag) were cloned into a pcDNA3.1(+) expression vector (Life Technologies) using BamHI and EcoRI. Mutated NPC2 constructs were then amplified using primers listed in the supplementary data and recloned into pcDNA3.1(+) with zeocin resistance (Life Technologies) to introduce a tag linker linked with a GSGGS amino acid linker sequence at the C terminus of human NPC2.

RNA interference and plasmid expression

CHO-F2 and 4-4-19-F2 cells were transfected with siRNA (Dharmacon, Lafayette, CO; sequences given in supplementary data) complexed to jetPRIME (Polyplus Transfection, New York, NY) at a final concentration of 50 nM following the manufacturer’s protocol. For cotransfections, 1 ng/µl plasmid DNA was added together with the siRNA. siRNAs against MLN64 and VDAC1 were predesigned against the respective murine genes and caused efficient silencing in CHO cells. To design siRNA against hamster NPC2, the cDNA sequence of hamster NPC2 was obtained by RT-PCR of CHO cell RNA with primers designed against murine Npc2 (supplementary data). Protein depletion was verified by immunoblotting of cell lysates using the following antibodies: rabbit anti-VDAC1 (ab15895, Abcam, Cambridge, MA), rabbit anti-MLN64 (PA1-562, ABR Thermo Fisher Scientific, Rockford, IL), goat anti-actin (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-NPC2, which recognizes human and hamster NPC2 (a generous gift from Peter Lobel, Rutgers University, Piscataway, NJ). Expression of flag-tagged mutant NPC2 was verified using an anti-flag antibody (M2, Sigma). In siRNA/plasmid DNA cotransfection experiments, cDNA was prepared using the FastLane Cell cDNA kit (Qiagen, Toronto, ON, Canada) and the expression levels of endogenous hamster NPC2 were determined by quantitative RT-PCR and the ΔΔct method using cyclophilin as a housekeeping gene. Expression of human flag-tagged NPC2 was verified by RT-PCR and agarose gel electrophoresis. Primer pairs used are listed in the supplementary data.

Cholesterol esterification

Cholesterol transport to the endoplasmic reticulum was measured as the incorporation of radiolabeled ['H]oleate into cholesterol esters in the presence of FBS (30, 31). CHO cells cultured overnight in LPDS containing medium were incubated for 4 h in medium containing FBS and ['H]oleate complexed to BSA. Cellular lipids were extracted with hexane/isopropanol (3:2, v/v), dried under nitrogen, separated by thin-layer chromatography,
and [3H]oleate incorporation into cholesterol esters and triglycerides was quantified by scintillation counting.

**Cholesterol transport to the mitochondrial inner membrane in intact cells**

Cholesterol transport to the mitochondrial inner membrane was measured as pregnenolone formation by F2 cells as described previously (11). In brief, following treatment as indicated, cells were incubated for 24 h in import medium consisting of phenol red-free, serum-free Ham’s F12/DMEM (1:1, v/v) containing 10 µM triostane to inhibit 3β-hydroxysteroid dehydrogenase. Where indicated, LDL was added to import medium. The pregnenolone concentration in import medium was measured by radioimmunoassay according to the manufacturer’s protocol (MP Biomedicals, Solon, OH) and reflects the amount of cholesterol transported to the mitochondrial inner membrane (11).

**Cholesterol transport to the mitochondrial inner membrane in semi-permeabilized cells**

CHO-F2 or 4-4-19-F2 cells grown to 80–90% confluency in Ham’s F12 medium (with treatments as indicated) were washed twice with transport buffer (25 mM Hepes-KOH, pH 7.2, 115 mM potassium acetate, 2.5 mM MgCl2) and incubated for 10 min at room temperature with 20 µg/ml digitonin in transport buffer. The semi-permeabilized cells were incubated for 20 min in transport buffer without digitonin, then rinsed twice to remove residual cytosol and digitonin. Cell permeabilization was verified by Trypan blue staining. To test the integrity of lysosomes and mitochondria, semi-permeabilized cells were incubated with LysoSensor Green and MitoTracker CMXRos (both from Life Technologies) and viewed with a Nikon TE2000 epifluorescence microscope using a 20× objective with excitation filters of 474/23 nm (LysoSensor) or 585/29 nm (MitoTracker), dual band dichroic, and a dual band emission filter of 572/42 nm (LysoSensor) or 585/29 nm (MitoTracker), dual band emission filters. Removal of cytosolic proteins was further verified using CHO cells stably expressing fluorescent mCherry protein. Following permeabilization as above, the levels of mCherry protein in transport buffer and cell lysates were analyzed by immunoblotting using a rabbit anti-red fluorescent protein antibody (Rockland Immunochemicals, Gilbertsville, PA). To measure mitochondrial cholesterol import, semi-permeabilized cells were incubated for times indicated at 37°C in transport buffer with 10 µM triostane, 0.8 µM malate, 0.8 µM pyruvate, and an ADP regenerating system (BD Biosciences, Mississauga, ON, Canada). Where indicated, 1 mM ATP, 250 µM GTP and an ATP regenerating system (10 µM phosphocreatine, 10 units/ml creatine kinase, 80 µM magnesium acetate) or 5 µM 22-R-hydroxycholesterol (22-OH-Chol) were added. Pregnenolone formation from the membrane-permeable precursor 22-OH-Chol reflects the mitochondrial steroidogenic capacity not limited by transport of cholesterol. Transport buffer was collected, centrifuged for 10 min at 800 g to remove cell debris, and extracted twice with diethylether. Lipid extracts were dried under nitrogen, and pregnenolone was measured by radioimmunoassay.

**Filipin staining**

Cells were stained with filipin to visualize unesterified cholesterol as described (32). Briefly, cells were fixed in paraformaldehyde, stained with 50 µg/ml Filipin III complex (Sigma), mounted in AquaMount (Thermo Fisher Scientific), and viewed with a Nikon TE2000 epifluorescence microscope using a 20× objective at filter settings of 387/11 nm (excitation) and 447/60 nm (emission).

**Statistical analysis**

For comparison of two groups, Student two-tailed t-test was used. For comparison of three or more groups, significance was calculated using ANOVA. Significance was assumed for P < 0.05.

**RESULTS**

**VDAC1 contributes to cholesterol delivery to mitochondria**

Our previous work showed that in CHO cells, depletion of the late endosomal transmembrane protein MLN64, but not depletion of NPC1, reduced cholesterol transport to the mitochondrial inner membrane (11). MLN64 contains a cytosolic, cholesterol-binding START domain homologous to StAR (23). One of the proteins found to interact with StAR to import cholesterol into mitochondria is VDAC1 in the mitochondrial outer membrane (9). We therefore measured the effects of depleting VDAC1 alone or in combination with MLN64 on mitochondrial cholesterol import, using CHO cells stably expressing a fusion protein of CYP11A1, adrenodoxin, and adrenodoxin reductase (F2) at the inner mitochondrial membrane (CHO-F2). In cells expressing F2, pregnenolone is formed specifically from cholesterol arriving at the mitochondrial inner membrane and thus measures mitochondrial cholesterol import (25, 26). Transfection of CHO-F2 cells with siRNA against VDAC1 efficiently depleted VDAC1 protein (Fig. 1A) and decreased pregnenolone formation by nearly 50% (Fig. 1B). Maximum rates of pregnenolone formation from the membrane-permeable precursor 22-hydroxycholesterol (22-OH Chol) were unchanged (107.4% ± 17% of siNT with 22-OH Chol), indicating that F2 enzyme activity was not affected. The decrease in pregnenolone formation in VDAC1-depleted cells was observed under two conditions of cholesterol homeostasis, namely, when CHO-F2 cells were deprived of cholesterol in lipoprotein-deficient serum (LPDS) before measurement of pregnenolone formation in the presence of LDL and when CHO-F2 cells were grown in full serum until measurement of pregnenolone formation in serum-free medium (Fig. 1B). The higher pregnenolone formation under refeeding conditions (LPDS→LDL) than during cholesterol deprivation (FBS→SF) was observed previously (11) and is in line with increased oxysterol formation in response to uptake of exogenous cholesterol (12). Thus, VDAC1 played a role in the transport of endosomal cholesterol to the mitochondrial inner membrane. The observation that cells transfected with siRNA against MLN64 (siMLN64) or against both VDAC1 and MLN64 (siVDAC/MLN64) produced less pregnenolone than cells depleted of VDAC1 alone (Fig. 1B) suggested that part of the cholesterol transported by MLN64 reached the mitochondrial inner membrane independently of VDAC1.

**NPC2 contributes to cholesterol delivery to mitochondria**

Next, we investigated cholesterol transport steps upstream of MLN64. According to a recent model, NPC2 mobilizes cholesterol from the inner membranes of multivesicular...
NPC2, but we did not investigate the role of NPC2 (11). However, NPC2 also transfers cholesterol from internal endosomal membranes into the endosomal perimeter membrane (15–17,19). We previously showed that cholesterol transport to mitochondria could bypass NPC1, but we did not investigate the role of NPC2 (11). To investigate whether NPC2 supplies cholesterol for transfer to mitochondria, we measured pregnenolone formation in CHO-F2 cells transfected with nontargeting siRNA (siNT) or siRNA against VDAC1 (siVDAC1), MLN64 (siMLN64), or a combination of both (siVDAC1/siMLN64) were incubated in medium containing serum (FBS) or lipoprotein-deficient serum (LPDS) for 24 h. Serum-free import medium containing no lipoproteins (SF) or LDL was added for 24 h, then collected and analyzed for pregnenolone. (A) Immunoblot of cell lysates probed for MLN64, VDAC1, and actin as a loading control. (B) Pregnenolone production measured by RIA and standardized to cell protein. Data are calculated as ng pregnenolone/mg protein/24 h and expressed as percentage of siNT. Data represent means ± SEM from three independent experiments in triplicate. *P < 0.05 by ANOVA.

Cells incubated with LPDS during the [3H]oleate pulse following cholesterol deprivation or incubated in serum-free medium following growth in FBS. Transfection with siRNA against NPC2 depleted NPC2 levels by approximately 80% (Fig. 2A) and caused endosomal cholesterol accumulation characteristic of NPC disease as shown by filipin staining (Fig. 2B). Pregnenolone formation and thus arrival of cholesterol at the mitochondrial inner membrane were decreased by nearly 50% in cells depleted of NPC2 under both conditions of cholesterol homeostasis (Fig. 2C). Depletion of both NPC2 and MLN64 (siNPC2/siMLN64) or of MLN64 alone (siMLN64) decreased pregnenolone formation to the same extent as depletion of NPC2 alone, suggesting that these two proteins may act in the same pathway. Pregnenolone formation was reduced to the same extent in CHO-F2 cells transfected with three different siRNA against NPC2 separately or a mixture of siRNA (Fig. 2D), indicating that the decrease was not due to an off-target effect of the siRNA. Thus, NPC2 contributed to cholesterol transport from endosomes to mitochondria.

Endosome-to-mitochondria transport of cholesterol requires the sterol-binding domain but not the NPC1 interaction domain of NPC2

Our observation that depletion of NPC2, but not depletion of NPC1, decreased mitochondrial cholesterol import was somewhat surprising, as cholesterol transport to ER and plasma membrane requires both NPC proteins and involves cholesterol transfer from NPC2 to the N-terminal, luminal loop of NPC1 (20–22). Several groups have described the sterol-binding domain of NPC2 (16,17) and mutations that interrupt sterol binding (21,33). In addition, a more recent study that investigated domains of NPC2 required for cholesterol transfer to NPC1 identified several NPC2 point mutants that had normal sterol-binding capacity but that displayed decreased cholesterol transfer to NPC1 (21). On the basis of these previous descriptions of NPC2 mutants, we generated flag-tagged expression vectors for human NPC2 carrying point mutations in the putative NPC1 interaction domain (NPC2 Y100A and NPC2 Y119S, corresponding to point mutations in V62 in mature NPC2) or in the sterol-binding domain (NPC2 Y81A, corresponding to a point mutation in Y100 in mature NPC2). CHO-F2 cells were cotransfected with siRNA against hamster NPC2 (siNPC2) or nontargeting siRNA (siNT) and expression vectors encoding wild-type or mutant human NPC2. Expression of human NPC2 was verified by RT-PCR (Fig. 3A). Quantitative RT-PCR with primers specific for hamster NPC2 indicated a downregulation of endogenous wild-type NPC2 by 50–60% (Fig. 3B), which was slightly less than was achieved by transfection with siRNA alone. Immunoblotting using an antibody that recognizes both hamster and human NPC2 verified that the different human NPC2 mutants were expressed at similar levels and not more than 2-fold higher than endogenous hamster NPC2 (Fig. 3C). To verify the function of our NPC2 constructs, we measured cholesterol transport to the ER (Fig. 3D). CHO cells cotransfected with the different human NPC2 expression vectors and with nontargeting siRNA or siRNA against hamster NPC2 were incubated with [3H]oleate in serum-containing medium for 4 h following an overnight incubation in LPDS medium. Under these conditions, formation of radiolabeled cholesterol ester reflects arrival of lipoprotein-derived cholesterol at the ACAT-accessible pool in the ER (30,31). Cells incubated with LPDS during the [3H]oleate pulse had much lower esterification rates than cells incubated in the presence of serum (Fig. 3D), demonstrating that esterification of [3H]oleate in FBS-fed cells reflected the transport of endocytosed cholesterol to the ER. As expected, transfection with siRNA against NPC2 (siNPC2) in combination with expression of an empty vector significantly reduced cholesterol esterification (Fig. 3D). Esterification rates were still higher than in cells incubated in LPDS without exogenous cholesterol, likely due to the residual NPC2 expression following siRNA/DNA cotransfection.
transfected cells. These results confirmed that mutations in the cholesterol-binding domain or the described NPC1-interaction domain of NPC2 inhibited the transport of lipoprotein-derived cholesterol to the ER (21).

Next, we tested whether NPC2 requires both cholesterol-binding and NPC1-interaction domains for mitochondrial cholesterol import. CHO-F2 cells were cotransfected as before with nontargeting siRNA (siNT) or siRNA against NPC2 (siNPC2), MLN64 (siMLN64), or a combination of both (siNPC2/siMLN64) were incubated in medium containing serum (FBS) or lipoprotein-deficient serum (LPDS) for 24 h. Serum-free import medium containing no lipoproteins (SF) or LDL was added for 24 h, then collected and analyzed for pregnenolone. (A) Immunoblot of cell lysates probed for MLN64, NPC2, and actin as a loading control. (B) Filipin staining showing unesterified cholesterol accumulation in siNPC2 cells. Scale bar: 50 µm. (C) Pregnenolone formation measured by RIA and standardized to cell protein. Data are calculated as ng pregnenolone/mg protein/24 h and expressed as percentage of siNT. (D) CHO-F2 cells were transfected with three different siRNA against hamster NPC2 separately (siNPC2-1, siNPC2-2, siNPC2-3) or together (siNPC2 pool). Pregnenolone formation was measured as above. Data represent means ± SEM from three independent experiments in triplicate. *P < 0.05 by ANOVA.
NPC2 restored pregnenolone formation, whereas expression of NPC2 Y119S with a mutation in the sterol-binding domain did not. In contrast to the sterol-binding mutant NPC2 Y119S, expression of NPC2 V81A or NPC2 V81D with a mutation in the NPC1-interaction domain restored pregnenolone formation to wild-type levels (Fig. 4). In line with our previous findings that endosomal cholesterol can be transported to mitochondria in the absence of NPC1 (11), these results demonstrated that transport of endosomal cholesterol to mitochondria relied on the sterol-transfer capacity of NPC2 but not on cholesterol transfer from NPC2 to NPC1.

Fig. 3. NPC2 proteins with point mutations in sterol-binding or NPC1-interaction domain cannot mobilize endosomal cholesterol to the ER. CHO cells were cotransfected with nontargeting siRNA (siNT) or siRNA against hamster NPC2 (siNPC2) and with plasmids encoding empty vector (Empty), human flag-tagged NPC2 (WT), or human flag-tagged NPC2 with a point mutation in the sterol binding domain (Y119S) or a point mutation in the NPC1-interaction domain (V81A and V81D). (A) RT-PCR analysis of expression of human flag-tagged NPC2 and cyclophilin. (B) Downregulation of endogenous NPC2 measured by quantitative RT-PCR calculated using the ΔΔct method with cyclophilin as housekeeping gene and standardized to siNT-Empty. Data are means ± SEM of one representative experiment in triplicate. (C) Immunoblot using antibodies that recognize human and hamster NPC2 and antibodies against tubulin as a loading control. Numbers below the blot are the ratio of total NPC2 to tubulin immunoreactivity standardized to siNT-Empty. (D) Cholesterol transport to the ER measured as cholesterol ester formation from [3H]oleate and LDL-derived cholesterol. Data are expressed as percent of siNT-empty and represent means ± SEM from two independent experiments in triplicate. *P < 0.05 by ANOVA; a Significantly different from the corresponding condition in siNT.

formation was measured in the presence of LDL following an overnight cholesterol deprivation. Pregnenolone formation was significantly decreased in cells transfected with siNPC2 and empty vector compared with cells transfected with nontargeting siRNA (Fig. 4). The decrease in pregnenolone formation was similar to the decrease in esterification rate in double-transfected cells (Fig. 3C). Expression of wild-type or mutant NPC2 had no effect on mitochondrial cholesterol import in CHO-F2 cells transfected with nontargeting siRNA (Fig. 4). In CHO-F2 cells depleted of endogenous NPC2, expression of wild-type NPC2 restored pregnenolone formation, whereas expression of NPC2 Y119S with a mutation in the sterol-binding domain did not. In contrast to the sterol-binding mutant NPC2 Y119S, expression of NPC2 V81A or NPC2 V81D with a mutation in the NPC1-interaction domain restored pregnenolone formation to wild-type levels (Fig. 4). In line with our previous findings that endosomal cholesterol can be transported to mitochondria in the absence of NPC1 (11), these results demonstrated that transport of endosomal cholesterol to mitochondria relied on the sterol-transfer capacity of NPC2 but not on cholesterol transfer from NPC2 to NPC1.
Endosomal cholesterol reaches the mitochondrial inner membrane in the absence of cytosolic carrier proteins and ATP

Our observations in intact CHO-F2 cells suggested that NPC2 transfers cholesterol from internal membranes to the perimeter membrane of late endosomes, from where it is transported to mitochondria with contributions by MLN64 and VDAC1, but not by NPC1. Next, we asked whether endosome-to-mitochondria movement of cholesterol required additional, cytosolic carrier proteins and/or passage through other subcellular membranes, such as ER or plasma membrane. To address these questions, we used CHO-F2 cells that were semi-permeabilized to remove cytosolic proteins following a protocol that was used previously to investigate endosome-to-trans Golgi cholesterol trafficking (34). Treatment with 20 μg/ml digitonin permeabilized the plasma membrane of 90% of cells, as shown by the uptake of Trypan blue (Fig. 5A). Retention of fluorescent LysoSensor Green and MitoTracker CMXRos added prior to digitonin treatment demonstrated that lysosomes and mitochondria maintained lumenal pH and membrane potential, respectively (Fig. 5A). In CHO cells stably expressing soluble mCherry protein, 95% of mCherry was removed with the digitonin-containing permeabilization buffer and the remaining mCherry with the following buffer exchange, demonstrating virtually complete removal of cytosolic proteins under our conditions of permeabilization (Fig. 5B). Transmembrane NPC1 and soluble luminal NPC2 were fully retained following digitonin treatment, showing the integrity of endosomes (Fig. 5B). Semi-permeabilized F2 cells incubated with 22-OH-Chol were able to synthesize pregnenolone (4717 ± 597 pg/hour/mg cell protein; means ± SEM, three independent experiments in quadruplicate), showing that the mitochondrial F2 protein remained functional. Mitochondrial energy substrates pyruvate and malate increased pregnenolone formation approximately 2-fold and were added to the transport buffer in all experiments (Fig. 5C). We then measured pregnenolone synthesis over 3 h following semi-permeabilization, with a full exchange of transport buffer every hour. After 3 h, pregnenolone synthesis per hour was still comparable to the initial pregnenolone formation after 1 h, indicating a sustained transport of cholesterol to and into mitochondria (Fig. 5C). Pregnenolone production from an excess of the membrane-permeable precursor 22-OH Chol was 2–3 times higher than production from endogenous cholesterol and constant over 3 h (Fig. 5D), indicating that the F2 enzyme remained active and that enzyme activity was not limiting for pregnenolone production from cellular cholesterol. Together, these findings showed that mitochondrial cholesterol import proceeded in the absence of cytosolic carrier proteins.

Endosomal cholesterol can be transported directly into mitochondria without passing through the endoplasmic reticulum

To determine whether cholesterol transported to the mitochondrial inner membrane in semi-permeabilized cells was derived from endosomes or other cellular cholesterol pools, such as plasma membrane or ER, we used 4-4-19-F2 cells, which have a loss-of-function mutation in NPC1 and are stably transfected with the F2 fusion protein (11). 4-4-19-F2 cells accumulate cholesterol in endosomes when grown in the presence of serum, but they slowly mobilize endosomal cholesterol when grown in the absence of exogenous cholesterol, as shown by filipin staining (Fig. 6A). Pregnenolone formation was three times higher in semi-permeabilized 4-4-19-F2 cells cultured in FBS than in 4-4-19-F2 cells depleted of endosomal cholesterol by incubation in LPDS medium prior to digitonin treatment (Fig. 6A). The addition of ATP/GTP and an ATP-regenerating system had no significant effect on pregnenolone formation, indicating that vesicular transport was not required (Fig. 6A). Pregnenolone formation was also decreased in 4-4-19-F2 cells transfected with siRNA against NPC2 prior to semi-permeabilization compared with cells transfected with nontargeting siRNA (Fig. 6B), demonstrating that cholesterol arriving at the mitochondrial inner membrane came from endosomes independently of NPC1 function.

As the plasma membrane was disrupted by digitonin and as transport of cholesterol from plasma membrane to mitochondria requires cytosolic carrier proteins (3), it seemed unlikely that endosomal cholesterol was transported first to the plasma membrane and then to mitochondria. To test whether endosomal cholesterol moved through the ER to mitochondria, we measured pregnenolone production in 4-4-19-F2 cells that were cholesterol-deprived overnight, incubated for 3 h in the presence or absence of LDL, and then semi-permeabilized with digitonin. In NPC1-defective 4-4-19 cells, transport of LDL-derived cholesterol to the ER is delayed, and little cholesterol reaches the ER within 3 h (31). In contrast, pregnenolone production increased after a 3 h addition of LDL (Fig. 6C), supporting the hypothesis that cholesterol
arriving at the mitochondrial inner membrane was endosome-derived and moved directly from endosomes to mitochondria without delivery to the ER. Together, the findings in semi-permeabilized cells showed that endosomal cholesterol trafficked directly into mitochondria without cytosolic carrier proteins or vesicular transport and that NPC2 contributed to this transport pathway even in the absence of functional NPC1.

**DISCUSSION**

Cholesterol is transported to the mitochondrial membranes for conversion to oxysterols and steroids and to maintain mitochondrial membrane structure and function. Several trafficking pathways supply mitochondria with cholesterol from different sources, including endosomes. In previous work, we showed that MLN64 contributes to mitochondrial cholesterol import, whereas NPC1 is not required for this transport pathway (11). Here, we have identified two additional proteins that play a role in mitochondrial cholesterol import, namely, NPC2 and VDAC1, and we show that cholesterol movement from endosomes to mitochondria i) is energy independent, ii) does not require cytosolic carrier proteins, iii) does not require an intact plasma membrane or transport to the ER, and iv) uses NPC2 but does not require interaction of NPC2 with NPC1.

To measure mitochondrial cholesterol import, we used CHO cells stably transfected with a GYP11A1 fusion protein (F2 protein) that converts cholesterol arriving at the mitochondrial inner membrane to pregnenolone, which is an established method to measure cholesterol transport to the mitochondrial inner membrane (25, 26). RNAi-mediated depletion of the mitochondrial outer membrane

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**Fig. 5.** Cholesterol is transported to the mitochondrial inner membrane in the absence of cytosolic carrier proteins. (A) Left panels: Phase images of 4-4-19-F2 cells stained with Trypan blue following incubation in the absence (control) or presence (digitonin) of 20 µg/ml digitonin. Dark staining of nuclei indicates permeabilization of plasma membrane. 20× objective, scale bar: 20 µm. Center and right panels: 4-4-19-F2 cells were stained with LysoSensor Green and MitoTracker Red CMXRos prior to incubation in the absence or presence of 20 µg/ml digitonin. Fluorescent images were acquired of unfixed cells using a 60× objective. Scale bar: 10 µm. (B) CHO cells stably expressing cytosolic mCherry protein were treated in the absence or presence of 20 µg/ml digitonin, and washed twice as described in Materials and Methods. Immunoblots of permeabilization buffer, wash buffers, and the lysates of remaining semi-permeabilized cells were probed with antibodies against NPC1, tubulin, mCherry, and NPC2. (C) Pregnenolone formation by semi-permeabilized CHO-F2 cells during 1 h in the absence or presence of 0.8 µM malate and 0.8 µM pyruvate. (D) Pregnenolone formation in F2-4-4-19 cells in the presence or absence of the membrane-permeable pregnenolone precursor 22-OH Chol over 3 h following semi-permeabilization. Transport buffer was fully replaced every hour. Data are calculated as pg pregnenolone per mg cell protein, and values for pregnenolone in transport buffer at the 2 h and 3 h time points were added to values from time points at 1 h and 2 h. Data represent means ± SEM of three independent experiments in triplicate.
channel VDAC1, late endosomal MLN64, and/or NPC2 in the lysosome lumen decreased pregnenolone formation over 24 h, suggesting a role for these proteins in mitochondrial cholesterol import (Figs. 1 and 2). VDAC1 is one of three isoforms that are responsible for the majority of anion, cation, and small metabolite exchange across the mitochondrial outer membrane and that play a central role in mitochondrial regulation of energy homeostasis and apoptosis. Several studies have described a connection between VDAC and cholesterol metabolism. Cholesterol affinity chromatography of mitoplasts from rat liver identified VDAC1 as a cholesterol-binding protein (35). Mitochondrial cholesterol levels are reduced in hepatoma cells depleted of VDAC1 (35). Moreover, VDAC1 forms a complex with StAR and the phosphate carrier protein and is required for StAR-mediated mitochondrial cholesterol import (9). StAR is homologous to the cytosolic domain of MLN64, suggesting that MLN64 and VDAC1 may also act in a common pathway of mitochondrial cholesterol import. This model is supported by our finding that depletion of VDAC1 and MLN64 did not further reduce pregnenolone formation compared with cells depleted of MLN64 alone (Fig. 1). However, because depletion of MLN64 or MLN64 and VDAC1 decreased mitochondrial cholesterol import to a greater degree than depletion VDAC1 alone (Fig. 1), not all MLN64-mediated cholesterol import requires VDAC1. The mechanism of VDAC1 involvement in mitochondrial cholesterol import is still unclear, but a direct transport of cholesterol through its ion channel seems unlikely.

On the endosomal side, depletion of NPC2 markedly decreased pregnenolone formation in CHO-F2 cells. NPC2 is a sterol transfer protein localized to the lumen of late endosomes/lysosomes (16, 18). Donor membranes containing lypobisphosphatidic acid, which is present in internal membranes of endosomes, increase sterol transfer rates of NPC2, suggesting that NPC2 mobilizes endosomal cholesterol by transferring it from internal membranes to the endosome perimeter (15, 16, 18, 19). In the absence of NPC2, cholesterol builds up in internal membranes and is not available for egress from the endosome. Our findings show that disruption of cholesterol transport at this step also affects cholesterol import into mitochondria (Fig. 2). Even though this finding is in agreement with NPC2 sterol transfer activity, it was surprising insofar as our previous work had shown that RNAi-mediated depletion of NPC1 or a loss-of-function mutation in NPC1 did not decrease cholesterol transport to the mitochondrial inner membrane in F2 cells (11). Deficiencies in either NPC1 or NPC2 cause lysosomal cholesterol accumulation and nearly indistinguishable phenotypes in human patients and animal models, indicating that these two proteins act in a common pathway (36). In vitro assays have demonstrated cholesterol transfer from NPC2 to the N-terminal luminal loop of NPC1 (20), and the region of NPC2 required for sterol transfer to NPC1 was mapped by alanine-scanning mutagenesis (21). A recent study showed NPC2 binding to the second luminal loop of NPC1 (22). Thus, the majority of cholesterol egress from endosomes to ER and plasma membrane involves cholesterol NPC2-mediated transfer of cholesterol from internal membranes to NPC1 in the perimeter membrane and subsequent distribution throughout the cell by an unknown mechanism (14, 21). To further investigate this apparent difference between transport of cholesterol to mitochondria and transport to the ER and plasma membrane, we generated expression vectors encoding human NPC2 with a point mutation in the sterol-binding domain (NPC2 Y119S), which...
is unable to bind or transfer cholesterol, and vectors encoding human NPC2 with a point mutation in the NPC1-interaction domain (NPC2<sup>V81A</sup> and NPC2<sup>V81D</sup>), which can bind cholesterol but not transfer it to NPC1 (21). As expected (21, 35), none of these NPC2 point mutants restored esterification of endocytosed cholesterol in CHO cells depleted of endogenous NPC2 (Fig. 3), demonstrating that sterol binding and interaction with NPC1 are required for transport of endosomal cholesterol to the ER. In contrast, NPC2<sup>V81A</sup> and NPC2<sup>V81D</sup> normalized pregnenolone formation, and only the sterol-binding mutant NPC2<sup>V1198</sup> was unable to compensate for loss of NPC2 in mitochondrial cholesterol import (Fig. 4). Thus, cholesterol transport to mitochondria relied on the sterol-binding capacity of NPC2, but not on cholesterol transfer to NPC1. Few other studies have identified biochemical differences between cells lacking NPC1 and cells lacking NPC2. Using dextran to follow membrane-impermeable lysosomal cargo, Goldman and Krise found that NPC1 was involved in the initial late endosome/lysosome fusion, whereas NPC2 was required for membrane fission from the nascent hybrid endosome/lysosomes and the release of lysosomal cargo-containing vesicles (37). Other studies have implicated NPC2 in the regulation of cell differentiation in adipocytes and fibroblasts, a role that has not been described for NPC1 (38, 39). Recently, Boadu et al. found that NPC2, but not NPC1, was required for ABCA1-mediated cholesterol mobilization from endosomes (40).

To determine whether endosomal cholesterol was transported directly to mitochondria or passed through other subcellular membranes and whether cytosolic carrier proteins were involved, we measured pregnenolone formation in semi-permeabilized F2 cells (Figs. 5 and 6). Pregnenolone formation was higher in F2 cells preincubated with full serum (i.e., high endosomal cholesterol and low cholesterol biosynthesis) than in cholesterol-deprived cells and proceeded at a steady rate for over 3 h after permeabilization (Fig. 5). However, addition of LDL for a time period shorter than required for transport to the ER still increased pregnenolone formation in NPC1-deficient F2 cells (Fig. 6), in line with a direct transport of endosomal cholesterol to mitochondria. Mitochondria acquire cholesterol from different sources by a variety of pathways, including carrier protein-mediated transport from the plasma membrane (3, 10) and transfer of cholesterol from the ER (11). The direct endosome-to-mitochondria cholesterol trafficking described here represents an additional pathway of cholesterol delivery to the mitochondrial inner membrane that may be part of the homeostatic response to cholesterol endocytosis through formation of oxysterols (12) and the regulation of mitochondrial membrane cholesterol. The accumulation of cholesterol in endosomes of NPC1-deficient cells, in which endosome-to-mitochondria cholesterol transport is possible, suggests that only a minor percentage of all endosomal cholesterol is transported directly to mitochondria. Nevertheless, as oxysterols and steroids are highly active signaling molecules and as even small changes in cholesterol content can have a relatively large effect on mitochondrial membrane properties, endosome-to-mitochondria trafficking is an important part of cellular cholesterol homeostasis. Moreover, in cells with endosomal cholesterol accumulation, this pathway may contribute to an increase in mitochondrial cholesterol, as has been observed in NPC1-deficient cells (11).

Together, a model emerges whereby most cholesterol is transported out of late endosomes to the ER and plasma membrane by transfer from internal membranes to NPC2 and on to NPC1. In addition, a small amount of endosomal cholesterol is transferred by NPC2 from inner membranes to the perimeter membrane, and from there to the mitochondrial outer and inner membranes by a pathway that involves MLN64 and VDAC1, but not NPC1<sup>B</sup>.

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