Lipid Homeostasis and Lipoprotein Secretion in Niemann-Pick C1-deficient Hepatocytes*

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Niemann-Pick C (NPC) disease is a fatal inherited disorder characterized by an accumulation of cholesterol and other lipids in late endosomes/lysosomes. Although this disease is considered to be primarily a neurodegenerative disorder, many NPC patients suffer from liver disease. We have investigated alterations that occur in hepatic lipid homeostasis using primary hepatocytes isolated from NPC1-deficient mice. The cholesterol content of Npc1+/− hepatocytes was 5-fold higher than that of Npc1+/+ hepatocytes; phospholipids and cholesteryl esters also accumulated. In contrast, the triacylglycerol content of Npc1−/− hepatocytes was 50% lower than of Npc1+/+ hepatocytes. We hypothesized that the cholesterol sequestration induced by NPC1 deficiency might inhibit very low density lipoprotein secretion. However, this process was enhanced by NPC1 deficiency and the secreted particles were enriched in cholesteryl esters. We investigated the mechanisms responsible for these changes. The synthesis of phosphatidylcholine, cholesteryl esters, and cholesterol in hepatocytes was increased by NPC1 deficiency and the amount of the mature form of sterol response element-binding protein-1 was also increased. These observations indicate that the enhanced secretion of lipoproteins from NPC1-deficient hepatocytes is due, at least in part, to increased lipid synthesis.

Niemann-Pick type C (NPC)2 disease is an inherited autosomal, recessive disorder that is characterized by an accumulation of cholesterol and other lipids in tissues. Individuals with this disease experience progressive neurodegeneration and premature death, typically during teenage years (1). The majority (∼95%) of cases of NPC disease result from mutations in the NPC1 gene (2), whereas the remaining 5% have mutations in the NPC2 gene (3). The NPC1 gene encodes a transmembrane protein that resides in late endosomes/lysosomes (4, 5). The protein contains a sterol-sensing domain, a leucine-zipper motif, and a lysosomal targeting sequence (2, 6, 7). In NPC1-deficient cells, low density lipoprotein (LDL)-derived cholesterol, as well as gangliosides and other lipids, accumulate in late endosomes/lysosomes. Thus, although the exact function of NPC1 has not yet been established, NPC1 appears to be required for the egress of cholesterol and/or other lipids from the endosomal pathway (8−13). A consequence of the sequestration of cholesterol in NPC1-deficient cells is that normal cholesterol homeostatic responses are impaired. In normal fibroblasts, when the cholesterol content increases, cholesterol biosynthesis is down-regulated and cholesterol esterification is stimulated (14). In NPC1-deficient fibroblasts, however, cholesterol becomes sequestered in late endosomes/lysosomes and the cholesterol homeostatic machinery in the endoplasmic reticulum (ER) fails to sense the increased level of cellular cholesterol. Consequently, cholesterol synthesis is inappropriately increased and the esterification of LDL-derived cholesterol is markedly decreased (15, 16).

In addition to the severe, progressive neurodegeneration in individuals with NPC disease, hepatomegaly and neonatal cholestasis occur in many NPC patients. A significant number of children with NPC disease die of liver failure within their first six months (17−20). Because the majority of cholesterol-containing LDLs are cleared from the circulation by the liver via receptor-mediated endocytosis (21, 22), NPC deficiency causes an accumulation of cholesterol in the livers of humans and mice to a greater extent than in any other tissue (22, 23). Mice lacking functional NPC1 represent an excellent model for studying NPC disease because they exhibit liver dysfunction, including hepatomegaly and elevation of plasma alkaline phosphatase and aminotransferase levels, similar to those seen in infants with NPC disease (20). In light of these liver problems, we have investigated alterations in lipid metabolism that occur in primary hepatocytes isolated from NPC1-deficient mice. Cholesterol levels in Npc1−/− hepatocytes are markedly (∼5-fold) higher than in Npc1+/+ hepatocytes and the rates of cholesterol esterification and the synthesis of cholesterol and phosphatidylcholine (PC) are increased. Moreover, NPC1 deficiency increases the amount of the mature form of sterol response element-binding protein (SREBP-1) and enhances the secretion of very low density lipoproteins (VLDLs) by hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—Hanks’ solution, collagenase, penicillin, streptomycin, and fetal bovine serum were obtained from Invitrogen. Phenylmethylsulfonyl fluoride, protein A-Sepharose CL-4B, tridecanoin standard for gas chromatography, phospholipase C from Clostridium welchii, and insulin were purchased from

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2 The abbreviations used are: NPC, Niemann-Pick type C; apo, apolipoprotein; CE, cholesteryl esters; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; LDL, low density lipoprotein; PC, phosphatidylcholine; SREBP, sterol response element-binding protein; TG, triacylglycerol; VLDL, very low density lipoprotein.
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Sigma, as was the inhibitor of acyl-CoA:cholesterol acyltransferase, Sandoz 58-035. Complete protease inhibitor tablets were from Roche. [3H]Glycerol (3 Ci/mmol), [1-14C]acetate (57 mCi/mmol), [2-14C]malonic acid lactone, [9,10-3H]oleic acid, and [methyl-3H]choline (75.7 Ci/mmol) were from Amer- sham Biosciences. All chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad. Goat anti-human apoli-ipoprotein (apo) B polyclonal antibodies were purchased from Chemicon (San Diego, CA). Mouse anti-ago IgG linked to horseradish peroxidase was obtained from Pierce. The rabbit anti-rat phosphatidylethanolamine N-methyltransferase-2 polyclonal antibodies were generated in our laboratory (24). The rabbit anti-human CTP:phosphocholine cytidylyltrans- ferase (CT) polyclonal antibodies (25) were generously pro-vided by Dr. S. Jackowski (St. Jude Children’s Research Insti- tute, Memphis, TN). The rabbit anti-canine calnexin antibodies were purchased from StressGen (Vancouver, British Columbia, Canada). The rabbit antibodies directed against human SREBP-1 and SREBP-2 were from Santa Cruz Biotech- nology Inc. (Santa Cruz, CA). Mouse anti-rabbit IgG linked to horseradish peroxidase was from Pierce. The chemilumines- cent reagent used for immunoblotting was from Amersham Biosciences. Cholesterol, cholesteryl esters (CE), triacylglycer- ols (TG), PC, and sphingomyelin used as thin layer chromatog- raphy standards were either purchased from Avanti Polar Lip- ids (Alabaster, AL) or isolated in our laboratory from rat livers. BiOCOAT collagen-coated cell culture dishes (60 mm) and thin layer chromatography plates (Silica Gel G, 0.25 mm thick- ness) were from VWR (Mississauga, ON, Canada), as was the Infinity Cholesterol reagent and the Triacylglycerol reagent used for detection of lipids in the column eluate from fast pro-tein liquid chromatography. All other chemicals and reagents were from Fisher Scientific or Sigma.

Culture of Primary Hepatocytes from Npc1-deficient Mice— Male mice (5 weeks old) were from a breeding colony of Balb/ cNctr-Npc1+/− mice established at the University of Alberta from original breeding pairs obtained from The Jackson Labo- ratories (Bar Harbor, ME). The mice were maintained under temperature-controlled conditions with a 12-h light, 12-h dark cycle, and were supplied with a 9% fat diet (number 5001 from Purina LabDiet, Richmond, IN) and water ad libitum. Hence- forth, mice homozygous or heterozygous for the Npc1 muta- tion are referred to as Npc1−/− and Npc1+/−, respectively, whereas wild-type mice are termed Npc1+/+. Because Npc1−/− mice do not produce offspring, Npc1+/− mice were used for breeding. The Npc1 genotype was determined from tail clippings by PCR analysis of genomic DNA using primers described previously (26).

For isolation of hepatocytes, mice were anesthetized by intra-peritoneal injection of Somnotol (22 μl/50 g body weight). A mid-line incision was made, and Hanks’ EGTA solution con- taining 1 mg/ml insulin was perfused through the portal vein until the liver was clear of blood. The upper and lower vena cava were tied and the perfusion was continued with Hanks’ colla- genase solution (100 units/ml) containing 1 mg/ml insulin until the liver became soft (~3 min). The liver was removed, cut into pieces, transferred to Hanks’ collagenase solution, and mixed until all clumps of tissue dispersed. The hepatocytes were washed three times in Dulbecco’s modified Eagle’s medium, then suspended in medium containing 10% fetal bovine serum and plated on collagen-coated dishes (1 × 10⁶ cells/ml). Cell viability (typically >90% for both Npc1+/+ and Npc1−/− hepatocytes) was estimated by trypan blue exclusion. After hepatocytes had attached to the dish (3–4 h), the medium was removed and cells were incubated in medium lacking serum.

Filipin Staining of Npc1+/+ and Npc1−/− Hepatocytes— Hepatocytes were cultured on collagen-coated coverslips for 16 h then fixed for 20 min at room temperature in 4% paraformaldehyde. Phosphate-buffered saline containing 10% goat serum and 50 μg/ml filipin was added for 2 h at room temperature. Pictures were taken using a Leica DM IRE2 fluorescence microscope (Leica, ON, Canada) with an excitation wavelength of 351 nm.

Mass of Lipids in Hepatocytes and Culture Medium—Hepatocytes from one 60-mm culture dish were scraped into water (1 ml) and lipids were extracted (27). The culture medium (2 ml) was also collected, cell debris was removed by centrifugation, and lipids were extracted (27). The amounts of TG, CE, and unesterified cholesterol were determined in hepatocyte lysates and in culture medium. After digestion of phospholipids with phospholipase C, tridecanoin (20 ng) was added as an internal standard. The mass of TG, cholesterol, and CE was determined by gas-liquid chromatography (26). For measure-ment of the mass of phospholipids, lipids were extracted from an aliquot of liver homogenate (2 mg of protein) or culture medium (2 ml) (27) and separated by thin layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:1, v/v). Bands corresponding to authentic PC and sphingomyelin were visualized by exposure of the plate to iodine and spots were scraped from the plate. Amounts of PC and sphingomyelin were quantified by phos- phorus analysis (28).

Isolation of VLDLs from Culture Medium and Measurement of Lipid Content—Npc1+/+ and Npc1−/− hepatocytes were incubated for 16 h in Dulbecco’s modified Eagle’s medium in the absence of serum. Culture medium (1.3 ml) was collected and mixed with 0.7 g of KBr. The sample was placed in a 5.0-ml Quick-seal tube and overlaid with 3.5 ml of 0.9% NaCl. The samples were centrifuged at 416,000 g for 1 h in a Beckmann VTi 90.0 rotor (29). Fractions containing high density lipopro- teins, LDLs, and VLDLs were sequentially collected from the bottom of the tube. The density of each fraction was deter- mined as the weight of 1.0 ml. Medium from 5 dishes of cells was combined. The mass of TG and CE was determined by gas-liquid chromatography.

ApoB Content of Hepatocytes, Culture Medium, and Plasma— Hepatocytes from one 60-mm culture dish were scraped into 750 μl of phosphate-buffered saline, then 250 μl of buffer contain- ing Tris-HCl (0.66 μ, pH 7.4), NaCl (0.75 μ), EDTA (25 mm), phenylmethylsulfonyl fluoride (5 mm), and Triton X-100 (5%, v/v) was added. Cellular extracts were centrifuged in a microcentrifuge at 14,000 × g for 10 min, after which proteins in the supernatant were immunoprecipitated by incubation overnight at 4 °C with anti-human apoB antibodies (7.5 μl). Protein A-Sepharose (45 mg) was added and the sample was mixed end-over-end for 2 h at 4 °C. The apoB-protein A com-
plexes were pelleted by centrifugation for 2 min at 14,000 × g in a microcentrifuge. For immunoprecipitation of secreted apoB, culture medium was centrifuged for 2 min at 1,000 × g to remove cell debris and anti-apoB antibodies were added to the supernatant. Equal amounts of protein in the immunoprecipitate were separated by electrophoresis on 5% polyacrylamide gels in the presence of 0.4% SDS, then transferred to polyvinylidene difluoride membranes. Proteins were immunoblotted with goat anti-human apoB antibodies and subsequently with anti-goat IgG linked to horseradish peroxidase. Immunoreactive proteins were detected by chemiluminescence. The amount of apoB was quantified by densitometric scanning of the blots and calculated as intensity of the band per mg of cell protein.

The apoB content of plasma was compared in Npc1+/+ and Npc1−/− mice that had been fasted for 16 h. Plasma (100 μl) was collected and lipoproteins were separated on the basis of density by ultracentrifugation on a KBr gradient (29). Ten fractions with densities ranging from 1.21 (fraction 10) to 1.006 g/ml (fraction 1) were collected. ApoB was immunoprecipitated from equal volumes of each fraction as described above, and proteins were separated by electrophoresis on 7.5% polyacrylamide gels containing 0.1% SDS. ApoB100 and apoB48 were analyzed by immunoblotting.

Separation of Lipoproteins on the Basis of Size from Hepatocyte Culture Medium—Hepatocytes were isolated from livers of male 5-week-old Npc1+/+ and Npc1−/− mice and incubated in Dulbecco’s modified Eagle’s medium for 16 h. Culture medium from four, 100-mm culture dishes was combined and concentrated to a volume of 200 μl from 10% fetal calf serum, then cholesterol esterification was assessed in human skin fibroblasts using the same protocol. Npc1+/+ fibroblasts were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ). The fibroblasts were grown to near confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, then cholesterol esterification was assessed as described above. In some experiments, cholesterol esterification was assessed in Npc1+/+ and Npc1−/− hepatocytes by incubation of cells with [3H]oleate for 2 h in the presence or absence of Sandoz 58-035 (2 μg/ml), an inhibitor of acyl-CoA:cholesterol acyltransferase.

**Immunoblotting of CTP-phosphocholine Cytidyltransferase, Phosphatidylethanolamine N-Methyltransferase, Calnexin, SREBP-1, and SREBP-2—Homogenates of livers from Npc1+/+ and Npc1−/− mice were centrifuged at 10,000 × g for 10 min to remove cell debris and mitochondria. The supernatant was centrifuged at 90,000 × g for 30 min to pellet membranes. Membrane proteins were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS, then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 10% skimmed milk, then incubated overnight with anti-CT antibodies (1:1,000 dilution) that recognize both CTα and CTβ; in hepatocytes the α isoform predominates. The membranes were incubated with anti-rabbit IgG linked to horseradish peroxidase (dilution 1:2,500). Immunoreactive proteins were visualized by enhanced chemiluminescence. Essentially the same procedure was used for immunoblotting of calnexin, SREBP-1, and SREBP-2, except that anti-calnexin antibodies were used at a dilution of 1:5,000, and SREBP-1 and SREBP-2 antibodies were used at a dilution of 1:250. For immunoblotting of phosphatidylethanolamine methyltransferase, proteins in liver homogenates were separated by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS, then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 10% skimmed milk, then incubated overnight with anti-phosphatidylethanolamine methyltransferase antibodies (dilution 1:1,000 dilution) followed by incubation with anti-rabbit IgG linked to horseradish peroxidase (dilution 1:2,500). Immunoreactive bands were visualized by enhanced chemiluminescence.

**Other Methods**—The protein content of samples was determined using the BCA protein assay (Pierce) with bovine serum albumin as standard. Statistical significance of difference was determined by the Student’s t test and differences were considered significant at p < 0.05.

**RESULTS**

**Accumulation of Lipids in NPC1-deficient Hepatocytes**—The distribution of unesterified cholesterol in primary hepatocytes isolated from 5-week-old Npc1+/+ and Npc1−/− mice was assessed by staining the cells with filipin (26, 31), an agent that

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FIGURE 1. Distribution of cholesterol is altered in Npc1−/− hepatocytes. Hepatocytes were isolated from livers of male 5-week-old Npc1+/+ (A) and Npc1−/− (B) mice, fixed in paraformaldehyde, and incubated with filipin. Filipin staining of unesterified cholesterol was detected by fluorescence microscopy. Size bar = 10 μm. C, lipids were extracted from Npc1+/+ and Npc1−/− hepatocytes and the unesterified cholesterol content was measured by gas-liquid chromatography. Data are mean ± S.E. of three independent experiments, each with triplicate analyses. *, p < 0.0001.

detects un-esterified cholesterol but not CE. Fluorescence microscopy of filipin-treated Npc1+/+ hepatocytes revealed that un-esterified cholesterol was localized mainly to the cell surface (Fig. 1A), whereas Npc1−/− hepatocytes showed an intense, punctate intracellular staining pattern, indicative of redistribution of unesterified cholesterol (Fig. 1B). These filipin staining patterns are reminiscent of those seen in other types of Npc1+/+ and Npc1−/− cells such as fibroblasts (32, 33), neurons (26), and glial cells (34). To establish the extent of unesterified cholesterol accumulation in NPC1-deficient hepatocytes, we measured the mass of cholesterol by gas-liquid chromatography. In Npc1−/− hepatocytes, the cholesterol content was ~5-fold higher than in Npc1+/+ hepatocytes (Fig. 1C). Thus, in hepatocytes, as in other types of cells, lack of functional NPC1 causes a pronounced re-distribution and accumulation of unesterified cholesterol. It is noteworthy that in mouse hepatocytes NPC1 deficiency causes a much greater accumulation of cholesterol than in other types of cells such as fibroblasts (25–50% increase) (35), glial cells (~20% increase) (34), and in cell bodies of neurons (~40% increase) (26).

The amounts of several other lipids, PC, sphingomyelin, CE, and TG, in Npc1+/+ and Npc1−/− hepatocytes were also compared. The amounts of PC (Fig. 2A), sphingomyelin (Fig. 2B), and CE (Fig. 2C) were significantly higher (by 48, 100, and 76%, respectively) in Npc1−/− hepatocytes than in Npc1+/+ hepatocytes. In contrast, the mass of cellular TG in Npc1−/− hepatocytes was 54% less (p < 0.001) than in wild-type hepatocytes (Fig. 2D). The cholesterol and CE content of livers of 5-week-old Npc1+/+ and Npc1−/− mice was also compared. The amount of unesterified cholesterol in NPC1-deficient livers was 5-fold higher than in wild-type livers (142.0 ± 24.9 versus 28.7 ± 3.9 μg/mg of protein and the amount of hepatic CE was not significantly changed by NPC1 deficiency (2.15 ± 1.41 versus 1.49 ± 0.33 μg/mg protein).

NPC1 Deficiency in Hepatocytes Increases the Secretion of Unesterified Cholesterol, CE, and Phospholipids, but Not TG—An important function of hepatocytes is to provide lipids for lipoprotein secretion. The majority of lipids secreted by cultured primary hepatocytes are associated with VLDLs although small amounts of cholesterol, CE, and sphingomyelin are also released into the medium as high density lipoproteins. PC, cholesterol, and sphingomyelin reside primarily on the surface monolayer of lipoprotein particles, whereas the neutral lipids TG and CE are in the core. The mass of cholesterol (Fig. 3A), PC (Fig. 3B), and sphingomyelin (Fig. 3C) secreted into the culture medium of NPC1-deficient hepatocytes was increased by NPC1 deficiency (by 117, 48, and 74%, respectively). In addition, the amount of CE in the culture medium of Npc1−/− hepatocytes was ~50% (p < 0.008) higher than in the medium of Npc1+/+ hepatocytes (Fig. 3D). However, the mass of TG secreted was unchanged by NPC1 deficiency (Fig. 3E).

These observations suggest that NPC1 deficiency in hepatocytes increases VLDL secretion. Furthermore, the data indicate that the composition of the neutral lipid core of the VLDLs might have been modified by NPC1 deficiency so that the particles contained an increased content of CE relative to TG. To confirm these conjectures, we isolated VLDLs from the culture medium of Npc1+/+ and Npc1−/− hepatocytes by density gradient ultracentrifugation and measured the mass of CE and TG by gas-liquid chromatography. The mass ratio of TG:CE in VLDLs from wild-type hepatocytes was 8.4 ± 2.1, whereas in VLDLs from Npc1−/− hepatocytes this ratio was 4.1 ± 1.2 (average ± S.D. from three independent experiments). Thus, NPC1 deficiency approximately doubles the proportion of CE relative to TG in the VLDL core.

Secretion of ApoB100-containing Lipoproteins Is Increased by NPC1 Deficiency—Each VLDL particle contains a single molecule of apoB (36). Because nascent VLDLs secreted by cultured hepatocytes are not internalized by the cells to a significant extent (37), the amount of apoB in hepatectomy culture medium...
reflects the number of VLDL particles secreted. To determine whether NPC1 deficiency increases the number of secreted VLDLs we analyzed by immunoblotting the amounts of apoB100 and apoB48 in culture medium of Npc1<sup>+/+ </sup>and Npc1<sup>−/− </sup>hepatocytes. During a 16-h incubation, ~80% more apoB100 was secreted by Npc1<sup>−/−</sup> hepatocytes than by Npc1<sup>+/+</sup> hepatocytes (Fig. 4A). The amount of apoB100 within the cells was similarly (by ~100%) increased (Fig. 4B). However, NPC1 deficiency did not alter the amount of either secreted (Fig. 4A) or cellular (Fig. 4B) apoB48. Therefore, NPC1 deficiency increases the number of apoB100-containing VLDLs secreted without increasing the secretion of apoB48-containing particles. Despite the enhanced secretion of apoB100 by Npc1<sup>−/−</sup> hepatocytes, the amount and density distribution of apoB100 in the plasma of Npc1<sup>−/−</sup> and Npc1<sup>+/+</sup> mice are similar (Fig. 4C). Normal levels of lipids and apoB in plasma have been previously reported in NPC1-deficient mice (22, 38). Thus, although NPC1 deficiency increases the secretion of VLDLs from hepatocytes, these lipoproteins appear to be removed from the circulation of Npc1<sup>−/−</sup> mice more efficiently than from Npc1<sup>+/+</sup> mice.

The experiments depicted in Figs. 3 and 4 demonstrate that the secretion of apoB100 and certain lipids into VLDLs is increased by NPC1 deficiency, but do not establish whether or not the total amount of lipid per VLDL particle, and consequently the size of the VLDLs secreted by Npc1<sup>+/+ </sup>and Npc1<sup>−/− </sup>hepatocytes, are the same. We, therefore, compared the size of the VLDLs using fast protein liquid chromatography. The density distribution of cholesterol (unesterified plus esterified) (Fig. 5A) and TG (Fig. 5B) was measured in the column eluate. The majority of VLDL-sized particles eluted after ~26 min, whereas low density lipoprotein-sized particles eluted after ~32 min, and high density lipoprotein-sized particles eluted after 42–50 min.
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Figures 4. ApoB100 secretion is increased by NPC1 deficiency. Hepatocytes were isolated from livers of Npc1+/+ and Npc1−/− mice and incubated for 16 h. ApoB was immunoprecipitated from the culture medium (A) and analyzed by polyacrylamide gel electrophoresis followed by immunoblotting. The amounts of apoB100 and apoB48 were quantified by densitometric scanning of the gels and are normalized to total cellular protein. Data show amounts of apoB secreted by Npc1−/− hepatocytes relative to amounts of apoB secreted by Npc1+/+ hepatocytes and are averages ± S.E. from at least three independent experiments. *p < 0.014; **p < 0.004. Inset, immunoblots of triplicate analyses of apoB100 and apoB48 in culture medium from Npc1+/+ and Npc1−/− hepatocytes, from one experiment representative of three independent experiments with similar results. C, plasma (100 μl) was collected from Npc1+/+ and Npc1−/− mice that had been fasted for 16 h. Lipoproteins were separated by ultracentrifugation. Ten fractions with densities ranging from 1.21 (fraction 10, bottom, containing high density lipoprotein-sized particles) to 1.006 g/ml (fraction 1, top, containing VLDL-sized particles) were collected. ApoB was immunoprecipitated and proteins were separated by electrophoresis on 7.5% polyacrylamide gels. ApoB100 and apoB48 were analyzed by immunoblotting. Representative immunoblots are shown from one of three independent experiments with similar results.

(Fig. 5, A and B). The elution time for TG and cholesterol in VLDLs secreted by Npc1+/+ and Npc1−/− hepatocytes was indistinguishable, demonstrating that the size of the VLDLs is not altered by NPC1 deficiency, despite changes in lipid composition of the particles.

A small amount of cholesterol is present in the medium from Npc1+/+ and Npc1−/− hepatocytes in high density lipoprotein-sized particles (Fig. 5A). In addition to normal-sized high density lipoproteins, a second population of particles, which were smaller in size than the high density lipoproteins generated by Npc1+/+ hepatocytes, was detected in the medium from Npc1−/− hepatocytes (Fig. 5A, arrow in lower panel). These smaller particles presumably contain less lipid than do the larger particles. This observation is consistent with the previous finding that the association of lipids with high density lipoproteins is impaired in NPC1-deficient fibroblasts (39, 40).

Thus, NPC1 deficiency in mouse hepatocytes increases the secretion of VLDLs containing apoB100. The size, and therefore the total amount of lipid, in the VLDLs is the same as in VLDLs secreted by wild-type hepatocytes. However, the composition of the neutral lipid core of VLDLs secreted by Npc1−/− hepatocytes is altered so that these particles contain more CE, and less TG, than do VLDLs secreted by Npc1+/+ hepatocytes.

Lipid Synthesis Is Increased by NPC1 Deficiency—To investigate the mechanism by which NPC1 deficiency increases VLDL secretion we compared the rates of lipid synthesis in Npc1+/+ and Npc1−/− hepatocytes because the supply of lipids is known to regulate VLDL secretion (reviewed in Ref. 41). PC synthesis was assessed by incubation of hepatocytes with [methyl-3H]choline, a substrate for PC synthesis via the CDP-choline pathway. The rate of incorporation of [3H]choline into PC in Npc1−/− hepatocytes (22.8 × 10−3 dpm/h/mg of protein) was approximately double that in Npc1+/+ hepatocytes (10.1 × 10−3 × dpm/h/mg of protein) (Fig. 6A); the amount of radiolabel taken up by the hepatocytes was the same for both NPC1 genotypes. Parallel radiolabeling experiments with [3H]glycerol and [14C]acetate showed that the incorporation of these radiolabeled precursors into PC was similarly increased by NPC1 deficiency (data not shown). These observations suggest that the rate of PC synthesis via the CDP-choline pathway is increased by NPC1 deficiency. In support of this idea, immunoblotting of hepatic PC revealed that the amount of CT, the rate-limiting enzyme of PC synthesis via the CDP-choline pathway (42), is markedly increased by NPC1 deficiency (Fig. 6B). Immunoblotting of calnexin, a resident protein of the ER, was used as a loading control. Interestingly, the amount of phosphatidylethanolamine N-methyltransferase protein, the enzyme that methylates phosphatidylethanolamine to PC in hepatocytes (43, 44), was unaffected by NPC1 deficiency (Fig. 6B). Thus, in NPC1-deficient hepatocytes the rate of PC synthesis, specifically via the CDP-choline pathway, is significantly enhanced, consistent with the increased amounts of cellular and secreted PC (Figs. 2A and 3B).

TG synthesis was examined in Npc1+/+ and Npc1−/− hepatocytes by incubation of the cells with [3H]glycerol. Fig. 6C shows that the incorporation of [3H]glycerol into TG after 1 h incubation is the same in Npc1−/− hepatocytes and Npc1+/+ hepatocytes. Similarly, the incorporation of [3H]oleic acid into TG was not significantly changed by NPC1 deficiency (data not shown). Thus, TG synthesis appears not to be significantly altered by NPC1 deficiency.

The synthesis of cholesterol was also examined in radiolabeling experiments. Npc1+/+ and Npc1−/− hepatocytes were incubated for up to 3 h with [14C]mevalonic acid lactone and radiolabel in cholesterol was quantified. The incorporation of radiolabel into cholesterol was modestly, but significantly, increased by NPC1 deficiency (Fig. 7A). A similar result was obtained when the hepatocytes were incubated with [13C]acetate.
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Our observation that in hepatocytes the amounts of cellular cholesterol (Fig. 2C) and secreted (Fig. 3D) CE were increased by NPC1 deficiency was surprising because a hallmark of NPC1 deficiency in LDL-loaded fibroblasts is a reduced availability of cholesterol for esterification by acyl-CoA:cholesterol acyltransferase (16). We, therefore, examined the esterification of cholesterol in Npc1−/− and Npc1+/− hepatocytes. First, we labeled the cells with [14C]mevalonic acid lactone and measured the incorporation of radiolabel into CE (Fig. 7B). Although the amount of label in CE was much lower than that in unesterified cholesterol (compare Fig. 7A), more [14C] was incorporated into CE of Npc1−/− hepatocytes than of Npc1+/− hepatocytes. We also examined cholesterol esterification by measuring the incorporation of [3H]oleate into CE. This method is widely used to assess the amount of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (45, 46). Fig. 7C shows that the incorporation of [3H]oleate into CE is severalfold higher in Npc1−/− hepatocytes than in Npc1+/− hepatocytes, suggesting that the pool of cholesterol available for esterification is higher in Npc1−/− hepatocytes than in Npc1+/− hepatocytes. We validated the use of this cholesterol esterification assay in our hands by demonstrating that cholesterol esterification in LDL-loaded Npc1−/− fibroblasts was, as expected, ~50% lower than in Npc1+/− fibroblasts (data not shown). Thus, NPC1 deficiency in hepatocytes increases cholesterol esterification.

We considered the possibility that the increased esterification of cholesterol in Npc1−/− hepatocytes occurred in cholesterol-laden late endosomes/lysosomes rather than via acyl-CoA:cholesterol acyltransferase. Nothdurft and co-workers (47) have shown that when cholesterol levels of endosomal/lysosomal membranes are high, as is the case in NPC1-deficient hepatocytes, cholesterol can be esterified in endosomes/lysosomes. We, therefore, performed the cholesterol esterification assay with [3H]oleate in the presence and absence of an inhibitor of acyl-CoA:cholesterol acyltransferase (Sandoz 50-035) (48). In Npc1−/− hepatocytes, the inhibitor partially (by ~33%) reduced the incorporation of [3H]oleate into CE (218 ± 19 dpm/mg of protein in the absence of inhibitor versus 149 ± 45 dpm/mg of protein in the presence of inhibitor). In Npc1+/− hepatocytes, the inhibitor reduced the incorporation of radiolabel into CE to a similar extent (~35%) (Fig. 7D), suggesting that the increased esterification induced by NPC1 deficiency is mediated by acyl-CoA:cholesterol acyltransferase.

NPC1 Deficiency in Hepatocytes Increases the Amount of Processed SREBP-1 but Not SREBP-2—When cholesterol becomes sequestered in late endosomes/lysosomes of NPC1-deficient cells (16, 32) the cells fail to sense the increased content of cholesterol and the normal regulation of cholesterol homeostasis is impaired (16, 35). For example, in NPC1-deficient fibroblasts pathways involved in cholesterol accretion (i.e. cholesterol synthesis and LDL receptor expression) are increased, whereas pathways involved in cholesterol removal (i.e. cholesterol esterification (16, 32, 35) and expression of the ATP-binding cassette protein, ABCA1 (39, 40)), are down-regulated. Many of these responses depend upon the proteolytic processing of two transcription factors, SREBP-1 and SREBP-2, that occurs in response to a reduced cholesterol content of the ER. Although SREBP-1 and SREBP-2 have some overlapping functions, the mature, nuclear form of SREBP-1 primarily promotes the expression of genes involved in fatty acid and glycerolipid synthesis, whereas SREBP-2 primarily increases expression of genes involved in cholesterol metabolism (49, 50). We, therefore, hypothesized that the increased secretion of VLDLs by Npc1−/− hepatocytes might have been, at least in part, the result of the increased activity of SREBP-1 and/or SREBP-2. Immunoblotting of proteins from mouse hepatocytes showed that the amount of mature SREBP-1 is increased by NPC1 deficiency (Fig. 8, A and B). No difference was observed in the amounts of the unprocessed form (125 kDa) of SREBP-1 in Npc1+/− and Npc1−/− hepatocytes (Fig. 8). These observations suggest that the increased amount of the mature form of SREBP-1 is due to increased processing, not an increased amount, of the unprocessed form of SREBP-1. Although the antibody we used does not distinguish between SREBP-1a and SREBP-1c, we attribute the increased amount of mature SREBP-1 primarily to SREBP-1c because this is the
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FIGURE 6. Radiolabeling of PC and TG in Npc1+/+ and Npc1−/− hepatocytes. A, Npc1+/+ and Npc1−/− hepatocytes were incubated for up to 3 h with [methyl-3H]choline. Lipids were extracted and PC was isolated by thin layer chromatography. Data are averages ± S.E. of three independent experiments, each performed in triplicate. B, lysates were prepared from Npc1+/+ and Npc1−/− hepatocytes and proteins were separated by polyacrylamide gel electrophoresis. The amounts of CT and phosphatidylethanolamine N-methyltransferase (PEMT) were assessed by immunoblotting. Calnexin was used as a control for equal loading of lanes of the gel. Duplicate analyses from one experiment are shown. The experiment was performed three times with similar results. C, Npc1+/+ and Npc1−/− hepatocytes were incubated for 1 h with [3H]glycerol. Lipids were extracted and TG was isolated by thin layer chromatography. Data are averages ± S.E. from three independent experiments, each performed in triplicate.

more abundant isoform in mouse liver (51). The amount of the processed form of SREBP-2 was not increased by NPC1 deficiency (Fig. 8). These data indicate that the mechanism by which VLDL secretion is increased in NPC1-deficient hepatocytes is by increased synthesis of CE, cholesterol and/or PC, and stimulation of lipogenesis induced by increased amounts of mature SREBP-1.

DISCUSSION

In these studies we have examined alterations in the synthesis of several lipids and VLDLs in response to NPC1 deficiency in hepatocytes isolated from the mouse model of NPC disease. Cholesterol levels in Npc1−/− hepatocytes from 5-week-old mice are ∼5-fold higher than in Npc1+/+ hepatocytes and the subcellular distribution of cholesterol is profoundly altered. The sequestration of unesterified cholesterol in NPC1-deficient hepatocytes does not, however, reduce VLDL secretion. In contrast, almost twice as many apoB100-containing VLDLs are secreted by Npc1−/− hepatocytes as by Npc1+/+ hepatocytes. In addition, NPC1 deficiency doubles the ratio of CE:TG in the VLDLs. The rate of synthesis of three important VLDL lipid components, PC, cholesterol, and CE, is significantly higher in Npc1−/− hepatocytes than in Npc1+/+ hepatocytes, and the amount of the mature form of SREBP-1 is increased. These data suggest that the increase in VLDL secretion induced by NPC1 deficiency is the result of increased lipid supply.

Lipid Synthesis in NPC1-deficient Hepatocytes—A hallmark of NPC1 deficiency is sequestration of LDL-derived cholesterol in late endosomes/lysosomes. The 5-fold higher level of cholesterol in Npc1−/−, compared with Npc1+/+, hepatocytes is more pronounced than in other types of NPC1-deficient cells (fibroblasts, astrocytes, and neurons) so far examined, in which the level of unesterified cholesterol is typically increased by 20–50% (26, 34, 35). The massive accumulation of cholesterol in NPC1-deficient hepatocytes is consistent with a report showing that in livers of 8-week-old Npc1−/− mice the cholesterol content was 10-fold higher than in livers from Npc1+/+ mice; the CE content of these livers was not reported. Moreover, the cholesterol content of Npc1−/− hepatocytes was recently reported to be ∼6-fold higher than that of Npc1+/+ hepatocytes (52). A likely explanation for the much larger accumulation of cholesterol in liver/hepatocytes compared with other NPC1-deficient tissues/cells is that the liver is responsible for the removal of the majority of LDLs from the circulation (21, 23, 53).

Our data demonstrate that in hepatocytes, as in fibroblasts (16), NPC1 deficiency increases cholesterol synthesis. Hepatocytes were incubated with serum (a source of LDLs) for the first 4 h of culture, then incubated without serum for the remainder of the experiment. In contrast, in intact mice, livers are continuously exposed to circulating LDLs. Despite presumed differences in lipid status between intact livers and isolated hepatocytes, our observations are consistent with studies in livers of 8-week-old Npc1−/− mice in which the rate of cholesterol synthesis was ∼50% higher than Npc1+/+ mice, and the level of 3-hydroxy-3-methylglutaryl-CoA reductase mRNA was doubled (20).

In LDL-loaded fibroblasts, cholesterol esterification is reduced by NPC1 deficiency (15, 35). Consequently, we were surprised to find that NPC1 deficiency in hepatocytes increased the rate of cholesterol esterification severalfold, and significantly increased the mass of cellular and secreted CE. One of the assays we used for measuring cholesterol esterification is widely used to assess the pool size of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (46, 54). It is noteworthy, however, that the cholesterol status of hepatocytes used in our experiments is likely to be different from that in fibroblasts in which cholesterol esterification is typically measured in cells that have been loaded with cholesterol from
In our studies, although the livers had been exposed to LDLs, the hepatocytes were plated in the absence of serum, the cells were subsequently incubated for up to 3 h with [1-14C]mevalonic acid lactone. Lipids were extracted and unesterified (A) and esterified (B) cholesterol were isolated by thin layer chromatography. Data are averages ± S.E. of triplicate analyses from four independent experiments. Cholesterol esterification was assessed as the enzyme that catalyzes PC formation via the methylation pathway, is unaltered by NPC1 deficiency. The mechanism by which CT protein is increased by NPC1 deficiency is not clear but expression of the gene encoding CT is probably not directly regulated by SREBP (57), although SREBP-1c increases fatty acid supply, which in turn, increases PC synthesis (58).

The rate of TG synthesis appears to be the same in Npc1−/− and Npc1+/+ hepatocytes and the mass of TG in NPC1-deficient hepatocytes is reduced by ~50%. The latter observation is in general agreement with recent reports showing that the amount of TG in livers of 8-week-old Npc1−/− mice is ~85% lower than in Npc1+/+ mice (20), and that in Npc1−/− hepatocytes the TG content is ~65% lower than in Npc1+/+ hepatocytes (52). The reason for the reduction in TG content of Npc1−/− hepatocytes and livers is not clear but is apparently not due to increased secretion of TG (Fig. 3E).

Secretion of VLDLs and Regulation of Lipid Homeostasis by NPC1-deficient Mouse Hepatocytes—The secretion of apoB by hepatocytes is regulated primarily post-transcriptionally by several mechanisms including lipid availability (reviewed in Ref. 41). ApoB is synthesized in excess of its requirement for VLDL secretion and apoB that is not secreted is degraded intracellularly (59). Our data show that NPC1 deficiency increases the number of VLDL particles secreted. Factors contributing to the increased secretion of VLDLs might be the increased availability of cholesterol, CE, and PC. PC synthesis is known to regulate VLDL secretion from primary hepatocytes. For example, inhibition of PC synthesis by either the CDP-choline or the methylation pathway reduces the secretion of apoB and VLDLs (30, 60, 61).

An additional factor that might enhance VLDL secretion from NPC1-deficient hepatocytes is the increased amount of the proteolytically processed form of the transcription factor SREBP-1 that we observed. Davis and co-workers (62) have shown that increased expression of SREBP-1 in hepatoma cells promotes transcription of the gene encoding CT is probably not directly regulated by SREBP (57), although SREBP-1c increases fatty acid supply, which in turn, increases PC synthesis (58).

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An additional factor that might enhance VLDL secretion from NPC1-deficient hepatocytes is the increased amount of the proteolytically processed form of the transcription factor SREBP-1 that we observed. Davis and co-workers (62) have shown that increased expression of SREBP-1 in hepatoma cells enhances apoB secretion. It is noteworthy, however, that increased availability of hepatic TG does not increase apoB secretion from primary rodent hepatocytes (59, 63, 64).

Although the mature form of SREBP-1 was increased by NPC1 deficiency in hepatocytes, the amount of the unprocessed form was not increased suggesting that proteolytic processing of SREBP-1 is increased by NPC1 deficiency. A similar increase in SREBP-1 processing has been observed in Npc1−/− fibroblasts (65). SREBP-1 primarily increases mRNAs encoded by genes involved in lipogenesis, whereas SREBP-2 mainly increases the expression of genes involved in cholesterol metabolism (e.g. 3-hydroxymethylglutaryl-CoA reductase and the LDL recep-
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A

Npc1 genotype

+/+

-/-

SREBP-1

125 kDa

68 kDa

SREBP-1

68 kDa

calnexin

90 kDa

B

SREBP-1, % of control

200

100

50

0

Npc1+/+

Npc1-/-

FIGURE 8. NPC1 deficiency increases the amount of the processed form of SREBP-1, but not SREBP-2. Lysates were prepared from Npc1+/+ and Npc1-/- hepatocytes. Proteins were separated by polyacrylamide gel electrophoresis and immunoblotted with antibodies directed against SREBP-1, SREBP-2, and calnexin (loading control). A, the unprocessed and processed forms of SREBP-1 and SREBP-2 (125 and 68 kDa, respectively) and the processed form of SREBP-2 (68 kDa) are indicated by arrows. Duplicate analyses from one experiment are shown. The experiment was performed 4 times with similar results. B, quantification of immunoblots of the mature form of SREBP-1 from densitometric scanning of the immunoblots from the 4 experiments described in A.

by NPC1 deficiency in the liver might provide a basis for reducing the severity of liver problems of children with NPC disease.

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