Centripetal cholesterol flow from the extrahepatic organs through the liver is normal in mice with mutated Niemann-Pick type C protein (NPC1)

Chonglun Xie, Stephen D. Turley, and John M. Dietschy[1]

Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235

Abstract **Niemann-Pick type C (NPC) protein functions to move unesterified cholesterol from the lysosomal compartment to other intracellular sites for further metabolism and/or excretion. This cholesterol is brought into the cell through the coated-pit pathway and accumulates in the lysosomes when NPC protein is mutated. The present study quantitated the alternative uptake process that brings cholesterol into the cell through the scavenger receptor, class B, type I (SR-BI) pathway in animals with this mutation. In homozygous NPC mice, the tissues of the extrahepatic compartment accumulated an excess of 14 mg of cholesterol each day per kg body weight, and synthesis increased by a similar amount (to 111 mg/day per kg) to compensate for this functional loss of sterol through lysosomal sequestration. An amount of cholesterol (108 mg/day per kg) nearly equal to that synthesized in the extrahepatic compartment was carried through the circulation by high density lipoprotein (HDL) and taken up by the liver. The rate of hepatic cholesterol excretion from the NPC mice as fecal acidic (65 mg/day per kg) and neutral (85 mg/day per kg) sterols was elevated 61% above control values and was accounted for by the total amount of cholesterol brought to the liver in HDL and synthesized in the hepatocytes. These studies demonstrated that while cholesterol entering tissues of the NPC animals through the coated-pit pathway became sequestered in the lysosomal compartment and was metabolically inactive, cholesterol that was newly synthesized or that entered cells through the SR-BI pathway was metabolized and excreted normally.**—Xie, C., S. D. Turley, and J. M. Dietschy. **Centripetal cholesterol flow from the extrahepatic organs through the liver is normal in mice with mutated Niemann-Pick type C protein (NPC1).** ***J. Lipid Res.*** **2000.** 41: **1278–1289.**

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The integrity of cells in virtually every tissue of the body, including neurons of the central nervous system, apparently depends on access to a continuous supply of cholesterol cycling through the cystosol to the plasma membrane. While several steps in this cycling process have been elucidated, only recently has the protein been identified and cloned that functions to move unesterified cholesterol from the lysosomal compartment to a metabolically active pool of sterol in the cell that can regulate cholesterol and bile acid synthesis, act as substrate for several biosynthetic pathways and be excreted from the cell into the plasma or bile (1–5). Because mutational inactivation of this molecule leads to the clinical syndrome of Niemann-Pick type C (NPC) disease, this protein has been designated NPC1. This molecule has an endoplasmic reticulum signal peptide, 13–16 potential membrane-spanning regions, and a possible lysosomal targeting motif (4–6). It manifests significant sequence homology to other cellular proteins such as PATCHED, SCAP, and HMG-CoA reductase that are also known to play important roles in maintaining intracellular cholesterol homeostasis (6–9). NPC1 is synthesized in the endoplasmic reticulum, moves through prelysosomal vesicles, and eventually enters the lysosomal compartment (10). There it is apparently crucial for transporting unesterified cholesterol, and probably other lipids, to sites within the cytosol where the sterol can be further metabolized and/or transported out of the cell (11).

Mutations that inactivate NPC1 have been described not only in humans, but also in mice, dogs, cats, and other species (4, 12–15). The phenotype is similar in all of these groups. Fetal growth and development, including development of the brain, apparently take place normally. However, shortly after birth progressive enlargement of organs such as the liver and spleen occurs, liver dysfunction becomes manifest, and progressive neurological degeneration supervenes (12, 15–17). This sequence of events takes place over a few years in the affected child but

Abbreviations: NPC, Niemann-Pick type C; NPC1, NPC protein; CM-C, cholesterol carried in chylomicrons; LDL-C, cholesterol carried in low density lipoproteins; LDLR, LDL receptor; LRP, LDLR-related protein; apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; SR-BI, scavenger receptor, class B, type 1.

[1] To whom correspondence should be addressed.

over only 5–10 weeks in the homozygous NPC mouse (16, 17). The availability of these animal models and, particularly, the mouse model, has provided a powerful tool for exploring the role of NPC1 in cholesterol homeostasis in the live animal, for dissecting the relative importance of different pathways for cholesterol movement across the cells of the different tissues, and for elucidating the mechanisms of how cholesterol accumulation may lead to cell death in organs like the liver and central nervous system.

The hallmark of the NPC mouse is the time-dependent accumulation of cholesterol in virtually every organ. While the newborn pup homozygous for this mutation has a whole-body cholesterol pool (2377 mg/kg body weight) that is barely elevated above that of control animals (1833 mg/kg), this pool progressively expands with age until at 7 weeks it is elevated 2.5-fold above the normal value (17). Every tissue, except the central nervous system, participates in this expansion, although there are marked differences in the level of sterol sequestration in different organs. For example, the concentration of cholesterol is elevated 10-fold in the liver but only 1.3-fold in striated muscle (17).

A series of observations supports the conclusion that in the whole animal in vivo, as in the fibroblast in vitro, this pool of sequestered unesterified cholesterol is derived from sterol entering the cells through the clathrin-coated-pit pathway. First, the rate of cholesterol accumulation in each tissue is proportional to the rate of uptake of sterol carried in either chylomicrons (CM-C) or low density lipoprotein (LDL-C) (17). Second, abrogation of LDL receptor (LDLR) activity alters the rate of cholesterol accumulation in each organ in a manner that parallels the observed change in uptake of LDL-C by that same tissue (18). Third, when the rate of CM-C cleared from the plasma is altered, there is a similar change in the rate of sterol accumulation in the liver, but not in other organs (17). Finally, the rate of uptake of CM-C and LDL-C from the plasma (86 mg/day per kg) is sufficient to account fully for the rate of cholesterol sequestration (67 mg/day per kg) observed in the whole animal (17, 18). Taken together, these quantitative findings are consistent with the view that in NPC disease unesterified and esterified cholesterol carried in CM and LDL is taken up into the various organs through interaction with either the LDL receptor (LDLR) or LDL-related protein (LPR) and then processed through the coated-pit pathway. The unesterified cholesterol that results from this processing, however, is unable to move out of the lysosomal compartment and provide sterol to meet the normal metabolic needs of the cells.

However, despite this major block in the flow of cholesterol through the coated-pit pathway, fetal development apparently occurs normally. The sterol required for such tissue growth and maintenance, therefore, must come from a source other than CM-C and LDL-C. Presumably, this second source is the cholesterol that is continuously synthesized in each cell and that moves out of the tissues of the extrahepatic compartment, through the plasma to the liver, and, ultimately, is excreted in the feces. Quantitatively, this centripetal flow of sterol from the sites of synthesis in the periphery to the intestinal lumen is the largest net flux of cholesterol found in every animal and equals about 11, 35, and 100 mg/day per kg, respectively, in species like the monkey, rabbit, and mouse (19–23). This flux begins with the movement of unesterified cholesterol from the cells of the extrahepatic organs to acceptors in the plasma such as apolipoprotein A-I (apoA-I), a process that may be facilitated by a specific transporter located in the plasma membrane or in the caveolae of these membranes (24–29). Once associated with apoA-I, the sterol is esterified by the enzyme lecithin:cholesterol acyltransferase and, in the rodent, this cholesteryl ester ultimately is selectively taken up into the liver and endocrine glands through interaction with the scavenger receptor, class B, type 1 (SR-BI) (30–34).

The present studies were undertaken to establish whether this HDL/SR-BI pathway is, in fact, functionally normal in animals with mutated NPC1. The first set of studies quantitated the rate of cholesterol acquisition in the tissues of the extrahepatic compartment. In a second set of investigations these rates were compared with independent measurements of the rate at which sterol moves through the plasma in HDL and is taken up by the liver and adrenal gland. Finally, a third set of experiments quantitated the utilization of this sterol for excretion as fecal acidic and neutral sterols. These quantitative measurements of the flow of cholesterol through the HDL/SR-BI pathway in normal and NPC mice, along with similar measurements of the flow of sterol through the coated-pit pathway (18), provide the first detailed description of cholesterol balance in an animal with mutation of NPC1.

MATERIALS AND METHODS

Animals, diets, and genotyping

Heterozygous NPC mice ($NPC^{+/-}$) with a BALB/c background were crossbred with homozygous LDLR knockout ($LDLR^{-/-}$) animals (18, 22). Three groups were ultimately generated that included mice that were $NPC^{+/+}/LDLR^{+/+}$, $NPC^{-/-}/LDLR^{+/+}$, and $NPC^{-/-}/LDLR^{-/-}$. The genotypes of these mice were identified by using either polymerase chain reaction (PCR) or Southern blot analysis (4, 17, 22, 35). After weaning, all animals were housed in plastic colony cages in rooms with alternating 12-h periods of light and dark. All mice were fed a basal rodent diet (No. 7001; Harlan Teklad, Madison, WI) containing 0.016% (w/w) cholesterol until they were studied at 7 weeks of age. In one experiment, either cholesterol (1%, w/w) or cholestyramine (2%, w/w) was added to the meal form of this basal diet. These supplemented diets were begun at the 6th week of age and were fed for 1 week. Most studies were carried out in the fed state during the final hour of the dark phase of the light cycle. In one preliminary experiment, rates of cholesterol synthesis were also measured in vivo at the mid-dark and mid-light points of the light cycle. All experimental groups contained nearly equal numbers of males and females, except for the experiment dealing with cholesterol balance in the adrenal, which was carried out using only male animals. All experimental protocols were approved by the Institutional Animal Care and Research Advisory Committee.

Isolation and radiolabeling of LDL and HDL

Mouse plasma was harvested from both male and female $NPC^{+/+}/LDLR^{-/-}$ mice maintained on the low-cholesterol basal rodent diet (22). The LDL and HDL fractions were isolated by

preparative ultracentrifugation in the density ranges of 1.020–1.055 and 1.063–1.21 g/mL, respectively. LDL was then radiolabeled with either ^{125}I-labeled tyramine cellobiose or ^{131}I (21, 22). The apoE-containing HDL that contaminated these LDL fractions was removed by passing the lipoprotein solutions over a heparin Sepharose CL-6B column (Pharmacia Biotech, Uppsala, Sweden) (36). After dialysis, these radiolabeled LDL preparations were passed through a 0.45-μm pore size Millex-HA filter immediately prior to injection into the recipient animal. HDL was labeled with either [1α, 2α(*n*)-^{3}H]cholesteryl oleyl ether or [4-^{14}C]cholesteryl oleate by exchange from donor liposomes (37–39). Freshly collected rabbit plasma was used as the source for cholesteryl ester transfer protein. Rabbit HDL accounted for <5% of the HDL in the reaction mixture. The labeled HDL was then reisolated by ultracentrifugation in the density range of 1.070–1.21 g/mL and dialyzed against isotonic saline. All four of these radiolabeled LDL and HDL fractions were used within 48 h of preparation.

Measurement of LDL and HDL clearance rates in vivo

Mice were anesthetized with diethyl ether and xylazine, and a catheter was inserted into a jugular vein. After awakening, each animal was given a bolus of ^{125}I-labeled tyramine cellobiose-labeled LDL followed by a continuous infusion of the same preparation at a rate determined to maintain a constant specific activity in the plasma (22, 40, 41). Ten minutes before the termination of the 4-h infusion period, a bolus of ^{131}I-labeled LDL was administered to each animal. The animals were exsanguinated at 4 h and tissue and plasma samples were then assayed for their content of ^{125}I and ^{131}I. A similar procedure was used for determining HDL clearance. Animals were administered a priming dose of [^{3}H]cholesteryl oleyl ether-labeled HDL followed by the continuous infusion of the same radiolabeled lipoprotein at a rate demonstrated to maintain a constant specific activity in the plasma over the next 4 h (39). Ten minutes before the end of the 4-h infusion period, each animal was administered the [^{14}C]cholesteryl oleate-labeled HDL. Plasma, tissue samples, and the remaining carcass were saponified in alcoholic KOH, and sterols were extracted and assayed for their ^{3}H and ^{14}C content. The rates of clearance of LDL and HDL were expressed as the milliliters of plasma cleared of its LDL or HDL content by each tissue each day per kilogram of body weight (ml/day per kg). The rates of cholesterol uptake by the various tissues from these two lipoproteins were calculated by multiplying these tissue clearance rates by the plasma LDL-C or HDL-C concentration, and these rates were expressed as the milligrams of lipoprotein cholesterol taken up by each organ each day per kilogram of body weight (mg/day per kg).

Measurement of cholesterol synthesis rates in vivo

Each animal was injected intraperitoneally with approximately 50 mCi of $^{3}H_2O$. One hour later the animals were anesthetized, exsanguinated, and the individual tissues were removed. These tissues and the remaining carcass were then saponified, and digitonin-precipitable sterols were isolated as described previously (42–44). The rates of sterol synthesis in each of these tissues were then expressed as the nanomoles of $^{3}H_2O$ incorporated into cholesterol per hour per gram (nmol/h per g). The rate of incorporation of $^{3}H_2O$ into sterols by the tissues was converted to an equivalent milligram quantity of cholesterol, assuming that 0.69 ^{3}H atoms was incorporated into the sterol molecule per carbon atom entering the biosynthetic pathway as acetyl-CoA. These rates were expressed as the milligrams of cholesterol synthesized each day per kilogram of body weight (mg/day per kg) (42, 44, 45).

Measurement of fecal acidic and neutral sterol excretion and cholesterol 7α-hydroxylase activity

Stools collected from individual mice over 72 h were dried, weighed, and ground. The rates of acidic and neutral sterol excretion were measured as previously described and were expressed as the equivalent milligrams of cholesterol excreted as fecal acidic or neutral sterols each day per kilogram of body weight (mg/day per kg) (46–48). The activity of cholesterol 7α-hydroxylase was measured in hepatic microsomal preparations by an HPLC method. This technique quantitated the mass of 7α-hydroxycholesterol formed from endogenous microsomal cholesterol after enzymatic conversion to 7α-hydroxy-4-cholesten-3-one, using cholesterol oxidase (49).

Measurement of plasma and tissue cholesterol concentrations

The total plasma cholesterol concentration was measured enzymatically (Kit No. 1127771; Boehringer Mannheim, Indianapolis, IN). Plasma lipoproteins were separated by simultaneously centrifuging plasma at densities of 1.020 and 1.063 g/mL. The cholesterol content in the top and bottom of each tube was quantitated by GLC, using stigmastanol (Sigma, St. Louis, MO) as an internal standard. The tissues and remaining carcass were saponified, and their sterols were extracted with petroleum ether. The total cholesterol concentration was determined by GLC (50).

Determination of tissue unesterified and esterified cholesterol concentration

Tissues were removed from animals and extracted in choloroform–methanol 2:1 (v/v). The unesterified and esterified cholesterol was separated on Sep-Pak Vac RC cartridges (Waters, Milford, MA) (51). The cholesterol was then quantitated by GLC.

Calculations

The data from all of these experiments were presented as mean values ± 1 SEM. The Student's unpaired *t*-test was used to compare the various sets of data for significant differences at the $P < 0.05$ level.

RESULTS

Effect of gender and light cycling on sterol balance in the mouse

As previous studies have shown that heterozygous NPC mice (NPC$^{+/-}$/LDLR$^{+/+}$) have no abnormalities in tissue cholesterol levels or rates of synthesis, these investigations were all carried out in homozygous animals (NPC$^{-/-}$/LDLR$^{+/+}$) (17). However, before beginning definitive measurements of cholesterol flow through the HDL/SR-BI pathway, preliminary experiments were necessary to establish the effect of gender and diurnal light variation on the different measurements of sterol balance in these mice. As shown in **Table 1**, both whole-body (Table 1A) and liver (Table 1B) weights were significantly lower in the females, compared to the males, of both genotypes. Brain size, however, was similar in the two genders (Table 1C). As has been reported before in the mouse (17, 22), the concentration of total cholesterol in the adrenal was nearly twice as high in the female NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$/LDLR$^{+/+}$ animals as in the respective males (Table 1D). Despite this finding, however, the whole-animal cholesterol pool in these 7-week-old mice was not significantly different in the males and females of either genotype (Table 1E). This finding reflected the fact that the

TABLE 1. Effect of gender on cholesterol pools and sterol turnover in NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$LDLR$^{+/+}$ mice

Metabolic Parameter	NPC$^{+/+}$/LDLR$^{+/+}$		NPC$^{-/-}$/LDLR$^{+/+}$	
	Male	Female	Male	Female
A. Weight of whole animal (g)	23.1 ± 0.3 (38)	19.6 ± 0.2 (42)[a]	18.5 ± 0.4 (42)	16.2 ± 0.3 (41)[a]
B. Weight of liver (g)	1.32 ± 0.04 (15)	1.11 ± 0.03 (21)[a]	1.39 ± 0.04 (22)	1.26 ± 0.05 (18)[a]
C. Weight of brain (g)	0.44 ± 0.01 (15)	0.45 ± 0.01 (24)	0.37 ± 0.00 (18)	0.38 ± 0.00 (21)
D. Cholesterol concentration in adrenal (mg/g)	26.4 ± 1.4 (15)	61.7 ± 4.5 (10)[a]	37.8 ± 2.0 (9)	65.7 ± 3.3 (12)[a]
E. Cholesterol pool in whole animal (mg/kg)	2,182 ± 57 (9)	2,308 ± 84 (9)	5,283 ± 196 (9)	5,659 ± 216 (8)
F. Cholesterol acquisition from diet (mg/day per kg)	29 ± 2 (5)	28 ± 2 (5)	23 ± 2 (6)	22 ± 1(5)
G. Cholesterol acquisition from synthesis (mg/day per kg)	126 ± 7 (10)	113 ± 7 (8)	165 ± 17 (7)	175 ± 7 (5)
H. Cholesterol excretion as fecal neutral sterols (mg/day per kg)	48 ± 3 (12)	37 ± 4 (9)	90 ± 8 (11)	78 ± 7 (6)
I. Cholesterol excretion as fecal acidic sterols (mg/day per kg)	41 ± 4 (9)	60 ± 4 (9)[a]	57 ± 4 (11)	79 ± 5 (6)[a]

Both the NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$/LDLR$^{+/+}$ mice used in this study were 7 weeks of age and had been maintained on the basal rodent diet since they were weaned. Each value represents the mean ± 1 SEM for the number of animals shown in parentheses.

[a] Values in the female groups that were significantly different ($P < 0.05$) from those in the male animals of the same genotype.

concentration of cholesterol in all of the other organs of the NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$/LDLR$^{+/+}$ animals was essentially the same in the males and females of these two groups (17). Importantly, the rates of cholesterol acquisition by the whole animal from the diet (Table 1F) and from de novo synthesis (Table 1G) were nearly identical in the males and females of each genotype. While the excretion of cholesterol from the whole animal as fecal neutral sterols also showed no gender effect (Table 1H), excretion as fecal acidic sterols was elevated approximately 40% in the NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$/LDLR$^{+/+}$ females, as compared with the respective male mice (Table 1I). Although not shown in Table 1, other studies similarly revealed no gender effect on the tissue uptake of either LDL-C or HDL-C. On the basis of these preliminary studies that showed no gender effect on nearly all of the major parameters of sterol balance, all subsequent quantitative measurements were carried out in groups of animals containing equal numbers of males and females of the two relevant genotypes. In this manner, even the small difference observed in fecal acidic sterol output was averaged out in the various experimental groups. Only in those studies dealing with cholesterol balance across the adrenal (Fig. 3) were male animals alone utilized.

Because rates of cholesterol acquisition depended critically on accurate measurements of rates of cholesterol synthesis in vivo over a 24-h period, a second preliminary experiment investigated the effect of light cycling in these mice on rates of sterol synthesis in the liver and in the organs of the extrahepatic compartment. As shown in **Fig. 1**, the rate of hepatic cholesterol synthesis per gram of tissue varied 3-fold between the mid-light and mid-dark periods of the light cycle in the liver of the control NPC$^{+/+}$/LDLR$^{+/+}$ mice (Fig. 1A). In contrast, there was no diurnal variation in the rate of sterol synthesis in the liver of the NPC$^{-/-}$/LDLR$^{+/+}$ animals or, most importantly, in the extrahepatic tissues of either of these genotypes (Fig. 1B). As there were significant differences in whole-animal and liver weights in these two groups of mice, these data were normalized to give absolute rates of cholesterol synthesis per hour per kilogram of body weight. Nevertheless, there was still a 3-fold variation in the rate of hepatic sterol synthesis in the NPC$^{+/+}$/LDLR$^{+/+}$ animals (Fig. 1C) These data also showed that the absolute rate of cholesterol synthesis in both the liver (Fig. 1C) and extrahepatic organs (Fig. 1D) of the NPC$^{-/-}$/LDLR$^{+/+}$ animals was significantly higher than seen in the NPC$^{+/+}$/LDLR$^{+/+}$ mice, as has previously been reported (17). While there was little diurnal variation in the rate of sterol synthesis in the tissues of the extrahepatic compartment, nevertheless, all subsequent measurements of the rate of cholesterol acquisition from de novo synthesis were made over the final 1-h interval at the end of the dark phase, just before the light phase began. Measurements at this time gave the most accurate, average value for the rate of cholesterol synthesis throughout the day and were used to estimate the rates of sterol synthesis in the various organs over a 24-h period.

Rates of cholesterol acquisition by the tissues of the extrahepatic compartment

The rate at which cholesterol moves through the HDL/SR-BI pathway ultimately is determined by the rate at which sterol is acquired by the organs of the extrahepatic compartment through uptake of LDL-C and de novo synthesis. The first set of experiments quantitated these two sources of cholesterol in the extrahepatic tissues. The characteristics of the 163 animals used in these measurements are outlined in **Table 2**. There were nearly equal numbers of males and females in the two genotypic groups. The whole-body weight of the NPC$^{-/-}$/LDLR$^{+/+}$ animals was significantly lower than that of the control group (Table 2A). However, when normalized to a constant whole-body weight of 1 kg, the weights of the brain (Table 2B) and the other extrahepatic tissues (Table 2D) were not significantly different in these two groups. In contrast, however, liver size was significantly greater in the NPC$^{-/-}$/LDLR$^{+/+}$ mice (77.0 g/kg) than in the control animals (55.3 g/kg), a finding characteristic of the mouse or child with NPC disease (16, 17). The pool of cholesterol in the whole body of the mutant mice was expanded 2.5-fold (~5,500 mg/kg) compared to the control ani-

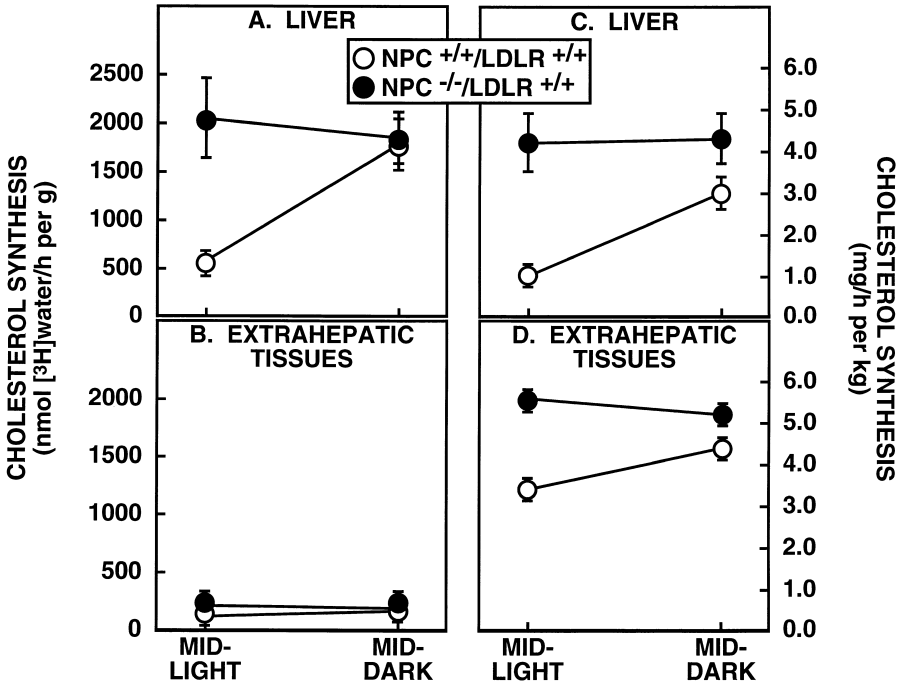

Fig. 1. Rates of cholesterol synthesis in the liver and extrahepatic tissues of 7-week-old animals measured over a 1-h interval at the mid-light and mid-dark points of the light cycle in $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ mice. (A and B) Rates expressed as the nanomoles of ^3H_2O incorporated into sterols each hour per gram of tissue. (C and D) These same data were used to calculate the milligrams of cholesterol synthesized each hour per whole organ after normalizing to a constant whole-body weight of 1 kg. Each value represents the mean ± 1 SEM for 9 or 10 animals in each group.

mals (~2,200 mg/kg) (Table 1E). In the tissues of the extrahepatic compartment, this pool equaled 1,636 ± 20 and 2,031 ± 35 mg/kg, respectively, in 1-day-old and 7-week-old $NPC^{+/+}/LDLR^{+/+}$ mice, and 2,240 ± 28 and 3,295 ± 88 mg/kg, respectively, in 1-day-old and 7-week-old $NPC^{-/-}/LDLR^{+/+}$ animals. Over the 7 weeks after birth, therefore, the control mice accumulated only 8 mg of cholesterol each day per kg body weight while the NPC animals stored 22 mg/day per kg. Thus, the $NPC^{-/-}/LDLR^{+/+}$ mice sequestered an excess of 14 mg/day per kg in the extrahepatic compartment.

While ~80% of the LDL pool is transported out of the plasma into the liver in both of these genotypes (18), the tissues of the extrahepatic compartment cleared this lipoprotein fraction at rates of 99 ± 7 and 104 ± 8 mL/day per kg, respectively, in the $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/$

TABLE 2. Rates of cholesterol acquisition in the extrahepatic tissues, cholesterol flux through the HDL/SR-BI pathway to the liver, and cholesterol excretion as neutral and acidic sterols in the feces of $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ mice

Metabolic Parameter	$NPC^{+/+}/LDLR^{+/+}$	$NPC^{-/-}/LDLR^{+/+}$
A. Weight of whole animal (g)	21.3 ± 0.3 (80)	17.4 ± 0.3 (83)[a]
B. Weight of brain (g/kg)	20.8 ± 0.1 (39)	21.7 ± 0.2 (39)
C. Weight of liver (g/kg)	55.3 ± 1.4 (36)	77.0 ± 1.7 (40)[a]
D. Weight of extrahepatic tissues (g/kg)	923.9 ± 40.5 (36)	901.3 ± 23.3 (40)
E. Extrahepatic cholesterol acquisition from LDL-C uptake (mg/day per kg)	7 ± 2 (9)	16 ± 3 (9)[a]
F. Extrahepatic cholesterol acquisition from synthesis (mg/day per kg)	85 ± 4 (10)	111 ± 5 (12)[a]
G. Hepatic cholesterol acquisition from HDL-C uptake (mg/day per kg)	100 ± 18 (8)	108 ± 18 (8)
H. Fecal neutral sterol excretion (mg/day per kg)	43 ± 3 (21)	85 ± 6 (17)[a]
I. Fecal acidic sterol excretion (mg/day per kg)	50 ± 4 (18)	65 ± 4 (17)[a]

Both the $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ mice used in these experiments were 7 weeks of age and had been maintained on the basal rodent diet since they were weaned. The weights of the brain, liver, and extrahepatic tissues have been normalized to a constant whole-body weight of 1 kg. Each value represents the mean ± 1 SEM for the number of animals shown in parentheses.

[a] Values in the $NPC^{-/-}/LDLR^{+/+}$ animals that were significantly different ($P < 0.05$) from those in the control $NPC^{+/+}/LDLR^{+/+}$ mice.

$LDLR^{+/+}$ animals. Because the LDL-C concentration in these same two groups equaled 7 ± 2 and 15 ± 2 mg/dL, the rates of cholesterol acquisition from LDL uptake in the control and mutant mice equaled 7 ± 2 and 16 ± 3 mg/day per kg, respectively (Table 2E). Thus, in the $NPC^{-/-}/LDLR^{+/+}$ animals, the rate of uptake of LDL-C (16 mg/day per kg), and, presumably, its accumulation in the cellular lysosomal compartment, fully accounted for the observed rate of sterol sequestration (14 mg/day per kg) in these extrahepatic tissues. The rates of cholesterol acquistion from de novo synthesis were much greater than from the uptake of LDL-C and equaled 85 ± 4 and 111 ± 5 mg/day per kg in the $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ animals, respectively (Table 2F).

From these measurements it could be predicted that in the control mice about 92 mg/day per kg of cholesterol should move from these extrahepatic organs into the plasma. In contrast, in the NPC mice most of the LDL-C that was taken up presumably was sequestered in the lysosomal compartment and accounted for the progressive expansion of the pool of unesterified cholesterol found in these extrahepatic tissues. This loss of sterol that was metabolically available to the cells, however, was compensated for by the significant increase in synthesis (Table 2F) so that in these mice approximately 111 mg/day per kg of cholesterol should move into the plasma space.

Rates of cholesterol movement through the plasma in HDL

To test the validity of these predictions, the second set of experiments was done to independently measure the rates of clearance of cholesterol from circulating HDL into the liver and adrenal, the major tissues involved in the net utilization and excretion of this sterol. As shown in **Fig. 2**, the clearance of cholesteryl esters from the plasma HDL pool equaled 120 ± 21 and 1.3 ± 0.1 mL/day per kg, respectively, in the liver and adrenal of the $NPC^{+/+}/LDLR^{+/+}$ mice, and these values were marginally lower in the NPC animals (Fig. 2A and B). However, because the concentration of cholesterol carried in HDL was slightly higher in the $NPC^{-/-}/LDLR^{+/+}$ mice (116 ± 2 mg/dL) than in the control animals (85 ± 7 mg/dL), the calculated rates of cholesterol uptake from this lipoprotein fraction into the liver equaled 100 ± 18 and 108 ± 18 mg/day per kg, respectively, in the control and mutant mice (Fig. 2C). Uptake of sterol by the adrenal also was nearly identical in the two genotypes (Fig. 2D). Thus, there was close agreement between the predicted rates of cholesterol movement out of the tissues of the extrahepatic compartment and the rates of uptake of cholesterol from HDL into the liver and adrenal. The critical question next addressed by the third set of experiments was whether this sterol could be utilized normally by these two organs for steroid hormone synthesis and for excretion as fecal acidic and neutral sterols.

Utilization of HDL-C and LDL-C by the adrenal

As the adrenal of the mouse expresses high levels of both SR-BI and LDLR activity (22, 39, 52) and is the only tissue in the NPC animals that maintains a pool of cholesteryl esters, this organ was utilized to explore the different fates of cholesterol taken up through these two pathways. As shown in **Fig. 3**, the rate of LDL-C uptake equaled only 0.25 ± 0.03 mg/day per kg in the control animals (Fig. 3A). This rate was similar in the $NPC^{-/-}/LDLR^{+/+}$ mice, but was reduced to only 0.04 ± 0.01 mg/day per kg in the $NPC^{-/-}/LDLR^{-/-}$ mice. The pool of unesterified cholesterol in the adrenal of these 7-week-old animals was in-

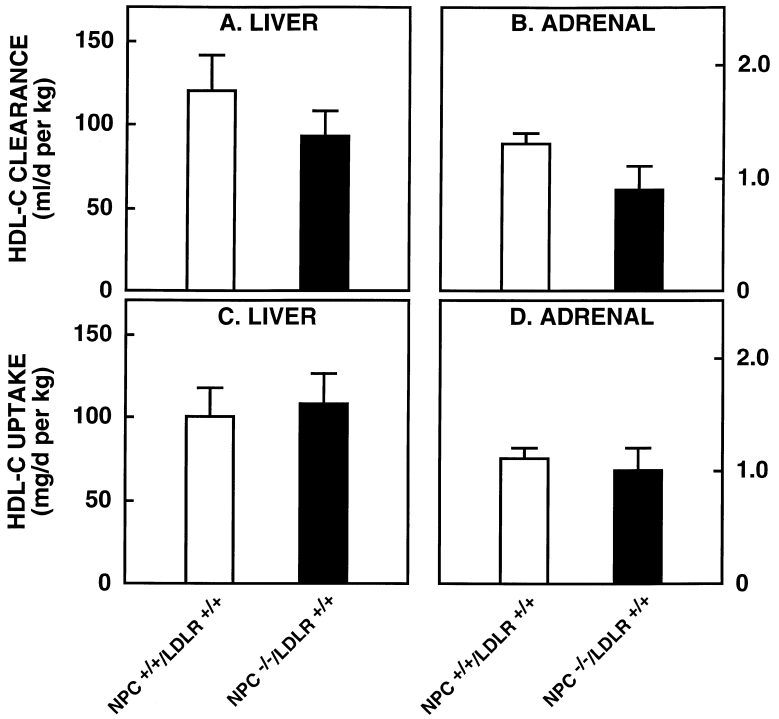

Fig. 2. Rates of clearance and uptake of cholesterol carried in HDL by the liver and adrenal of 7-week-old $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ mice. (A and B) Rates of uptake into these two organs expressed as the milliliters plasma cleared of cholesterol in this lipoprotein each day per kilogram of body weight. These clearance values were multiplied by the concentration of HDL-C in each group of animals to yield the milligrams of cholesterol taken up each day per kilogram of weight, and these values are shown in (C) and (D). Each value represents the mean ± 1 SEM for 8 animals in each group. There were no significant differences ($P < 0.05$) between values found in the $NPC^{+/+}/LDLR^{+/+}$ and $NPC^{-/-}/LDLR^{+/+}$ animals.

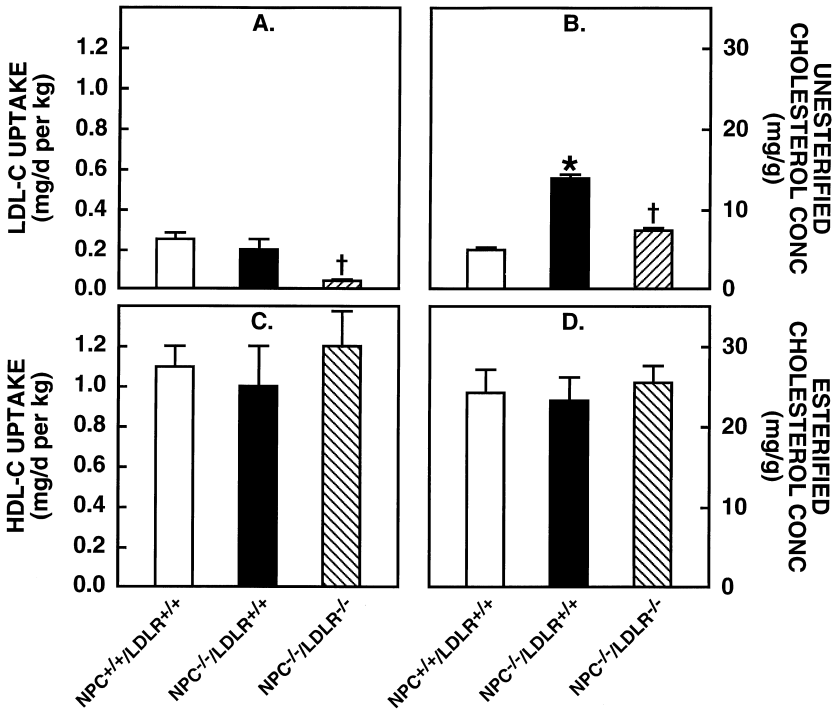

Fig. 3. Rates of LDL-C and HLD-C uptake and the concentration of unesterified and esterified cholesterol in the adrenals of $NPC^{+/+}/LDLR^{+/+}$, $NPC^{-/-}/LDLR^{+/+}$, and $NPC^{-/-}/LDLR^{-/-}$ mice. Only male animals were used in this experiment. Each value represents the mean ± 1 SEM for 6–8 mice in each group. The asterisk identifies those values in the $NPC^{-/-}/LDLR^{+/+}$ group that were significantly different ($P < 0.05$) from those in the control $NPC^{+/+}/LDLR^{+/+}$ animals while the dagger identifies those values in the $NPC^{-/-}/LDLR^{-/-}$ mice that were significantly different from those in the $NPC^{-/-}/LDLR^{+/+}$ mice.

creased from 4.9 ± 0.13 mg/g in the control mice to 13.9 ± 0.5 mg/g in the NPC animals. However, with abrogation of LDLR activity, this sequestered pool of unesterified cholesterol was markedly reduced in direct proportion to the observed decrease in LDL-C uptake in the $NPC^{-/-}/LDLR^{-/-}$ animals (Fig. 3B). Thus, while relatively little LDL-C was taken up into the adrenal of the mouse, this sterol apparently fully accounted for the pool of excess unesterified cholesterol sequestered in the coated-pit pathway of this organ.

In contrast, the uptake of cholesterol from HDL was nearly 5-fold greater than from LDL in the adrenal of the control animals, and this rate was essentially unaffected by elimination of either NPC1 or LDLR activity (Fig. 3C). This finding, in turn, presumably accounted for the fact that the pool of cholesteryl esters in this organ was maintained in both the $NPC^{-/-}/LDLR^{+/+}$ (23 ± 3 mg/g) and $NPC^{-/-}/LDLR^{-/-}$ (26 ± 2 mg/g) animals at levels essentially equal to those seen in the $NPC^{+/+}/LDLR^{+/+}$ (24 ± 3 mg/g) controls. Furthermore, the concentration of circulating corticosterone was actually elevated in the $NPC^{-/-}/LDLR^{+/+}$ mice (383 ± 38 ng/mL) compared to the control animals (120 ± 17 ng/mL). Thus, in contrast to LDL-C taken up through the coated-pit pathway, HDL-C taken up through the SR-BI mechanism apparently could maintain the adrenal pool of cholesteryl esters which, in turn, could be utilized for steroid hormone production.

Utilization of HDL-C by the liver for the formation of fecal acidic and neutral sterols

Of much greater quantitative importance is the ability of the liver to metabolize and excrete cholesterol taken up from HDL. Previous studies had shown that the large pool of unesterified sterol stored in this organ could be accounted for by the uptake and sequestration in the coated-pit pathway of cholesterol carried in LDL and CM (17, 18). Thus, in the NPC mice, cholesterol converted to bile acids or excreted into the intestinal lumen must come from sterol that is either taken up from HDL or that is newly synthesized in the liver. As shown in Table 2, the rate of neutral sterol excretion was twice as high in the $NPC^{-/-}/LDLR^{+/+}$ (85 mg/day per kg) mice as in the control (43 mg/day per kg) animals (H) and, importantly, even fecal acidic sterol excretion was marginally elevated (65 vs. 50 mg/day per kg) in the mutant animals. Because the liver of the $NPC^{+/+}/LDLR^{+/+}$ mice synthesized only 30 ± 5 mg of cholesterol each day per kg, and a portion of this was used for the assembly of very low density lipoproteins, most fecal sterols must have come from cholesterol reaching the liver in HDL. Similarly, even though synthesis in the liver of the $NPC^{-/-}/LDLR^{+/+}$ mice was elevated nearly 70%, the great majority of fecal sterols in the mutant animals must also have come from the uptake of HDL-C. Thus, in the NPC mice, the liver, like the adrenal, was apparently able to fully utilize cholesterol from HDL, presumably taken up through the SR-BI pathway, for secretion in bile or for the synthesis of bile acids.

Substrate specificity and regulation of bile acid synthesis in the liver of NPC mice

A final set of experiments was undertaken to provide further support for the concept that in the NPC mice cholesterol entering the liver through the SR-BI pathway had a different metabolic fate than sterol entering through the coated-pit pathway. In the mouse, the rate of bile acid synthesis is known to be regulated by both cholesterol acting through the LXRα nuclear receptor and bile acid acting through the FXR receptor (53–55). The response of the

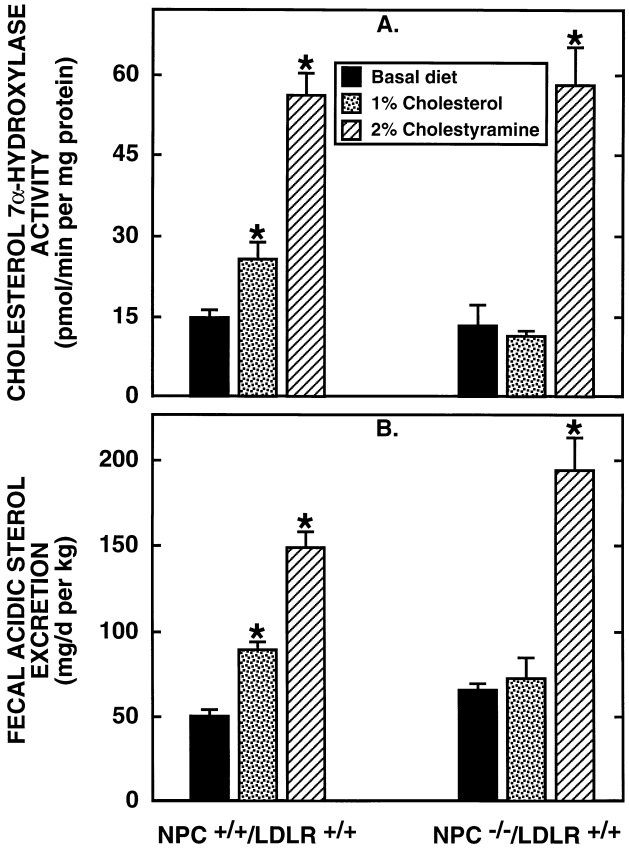

Fig. 4. Activity of cholesterol 7α-hydroxylase and rates of fecal acidic sterol excretion in NPC$^{+/+}$/LDLR$^{+/+}$ and NPC$^{-/-}$/LDLR$^{+/+}$ mice fed cholesterol or cholestyramine. Beginning at 6 weeks of age, groups of mice were either maintained on the basal rodent diet or placed on this same diet to which was added either 1% cholesterol or 2% cholestyramine. These measurements were made 1 week later. Mean values ± 1 SEM are shown for 6–8 animals in each group. The asterisks identify those values in each genotype that were significantly different from the values observed in the mice fed the basal rodent diet alone.

NPC mice to these two mechanisms of regulation is shown in **Fig. 4**. In the NPC$^{+/+}$/LDLR$^{+/+}$ animals, the rates of both cholesterol 7α-hydroxylase and fecal acidic sterol excretion were significantly increased in response to feeding dietary cholesterol or cholestyramine (Fig. 4A and B). In contrast, the NPC$^{-/-}$/LDLR$^{+/+}$ mice did not respond to dietary sterol even though the concentration of unesterified cholesterol in the liver of these animals became significantly elevated (17). The rate of cholesterol 7α-hydroxylase and fecal bile acid excretion did increase appropriately, however, after feeding cholestyramine.

While this experiment suggested that the unesterified cholesterol sequestered in the coated-pit pathway could not signal the LXRα nuclear receptor in the NPC mice, the question remained as to whether this pool of sequestered sterol could be used for bile acid synthesis, particularly under circumstances where cholesterol 7α-hydroxylase activity was markedly increased by activating the FXR nuclear receptor. As shown in **Table 3**, cholestyramine feeding in the NPC$^{-/-}$/LDLR$^{+/+}$ mice increased the excretion of acidic sterols nearly 3-fold, from 68 to 185 mg/day per kg (Table 3A), yet there was no reduction in the pool of unesterified cholesterol in the liver of these two groups of animals (Table 3C). Importantly, however, the increment in bile acid synthesis (117 mg/day per kg) brought about by cholestyramine feeding was closely matched by a parallel increase in the rate of hepatic cholesterol synthesis (132 mg/day per kg). Thus, in NPC disease, cholesterol reaching the liver in the coated-pit pathway could not act as a regulator of bile acid synthesis nor could it be utilized as substrate for this degradative pathway. In contrast, cholesterol reaching the liver through the SR-BI pathway or synthesized locally could maintain sterol balance across this organ in a normal manner.

DISCUSSION

These studies provide strong evidence that, in the presence of mutated NPC1, cholesterol newly synthesized in the tissues of the extrahepatic compartment moves normally through the plasma carried in HDL, is taken up through the SR-BI transporter into the adrenal and liver, and is utilized for the synthesis of steroid hormones and bile acids and for excretion into the feces. In contrast, cholesterol entering these tissues through the coated-pit pathway cannot regulate intracellular events, act as a substrate for bile acid formation or be excreted into the feces. This sterol, therefore, is sequestered in the cells of the var-

TABLE 3. Rates of bile acid and cholesterol synthesis and the concentration of cholesterol in the liver of NPC$^{-/-}$/LDLR$^{+/+}$ mice fed cholestyramine

	Diet	
	Basal	Cholestyramine
A. Fecal acidic sterol excretion (mg/day per kg)	68 ± 5 (7)	185 ± 20 (7)[a]
B. Hepatic cholesterol synthesis (mg/day per kg)	58 ± 5 (6)	190 ± 21 (6)[a]
C. Hepatic unesterified cholesterol concentration (mg/g)	20 ± 2 (7)	21 ± 3 (7)

NPC$^{-/-}$/LDLR$^{+/+}$ mice 6 weeks of age were given either the basal rodent diet or this same diet containing cholestyramine (2%, w/w). One week later the rates of fecal acidic sterol excretion and hepatic cholesterol synthesis were measured, and the concentration of unesterified cholesterol in the liver was quantitated. Each value represents the mean ± 1 SEM for the number of animals shown in parentheses.

[a] Values in the cholestyramine-fed animals that were significantly different ($P < 0.05$) from those in the animals fed the basal diet alone.

ious tissues and accounts for the progressive expansion of the whole-body pool of cholesterol that is characteristic of the mouse with homozygous NPC disease. The quantitative measurements obtained in this study, along with those reported earlier (18), provide a very complete picture of the handling of cholesterol through these two pathways in 7-week-old, homozygous NPC mice. These measurements are summarized in **Fig. 5**.

As illustrated by the dark arrows, the rates of clearance of CM-C and LDL-C in the NPC$^{-/-}$/LDLR$^{+/+}$ mice are not significantly different from those in control animals (18). Uptake of these particles through the coated-pit pathway delivers about 70 mg of cholesterol each day per kg to the liver and 16 mg each day per kg to the tissues of the extrahepatic compartment. The delivery of these amounts of sterol through the coated-pit pathway fully accounts for the observed rate of sterol accumulation in the whole animal (67 mg/day per kg) and for the rate of expansion of this pool in the organs of the extrahepatic compartment (22 mg/day per kg). These findings are consistent with the earlier observations in NPC fibroblasts that cholesterol entering cells through the coated-pit pathway becomes trapped in an intracellular compartment consisting of late endosomes and lyosomes (3, 10, 11). Since this trapped cholesterol apparently is not metabolically available to the cells, this sequestration, in effect, is tantamount to the net loss of 67 mg/day per kg of cholesterol from the body.

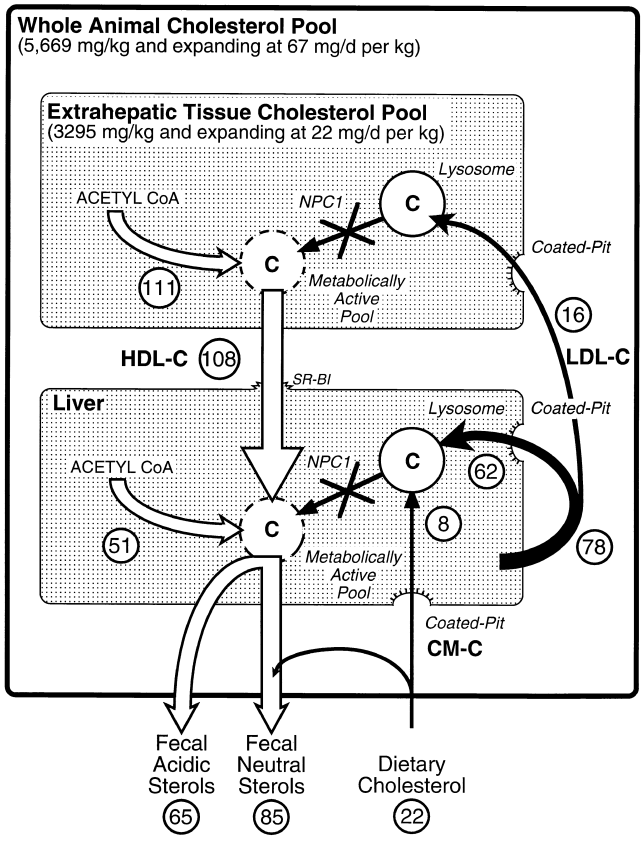

Fig. 5. Diagrammatic representation of the net flow of cholesterol through the various pathways and tissues of the NPC mouse. The pools of cholesterol are shown for the whole animal and extrahepatic tissues. The dark arrows represent the flow of cholesterol carried in chylomicrons and LDL that moves into the various tissues through the coated-pit pathway, while the open arrows delineate the movement of cholesterol from the sites of synthesis through the HDL/SR-BI pathway. The solid circles denote the pools of cholesterol (C) contained within the lysosomal compartment while the dashed circles represent the pools of cholesterol metabolically available in the cells that can regulate various biosynthetic pathways, act as substrate for these pathways or be excreted. The circled numbers represent the milligrams of cholesterol moving through each pathway each day per kilogram of body weight (mg/day per kg) and are taken from the data presented in this paper or from studies reported elsewhere (18).

The NPC animals respond to this net loss of functional cholesterol by simply increasing the rate of sterol synthesis in the whole animal by a nearly equal amount (from 120 mg/day per kg in control animals to 182 mg/day per kg in the NPC$^{-/-}$/LDLR$^{+/+}$ mice) (17). In the tissues of the extrahepatic compartment, synthesis increases to 111 mg/day per kg to just offset the amount of sterol entering the sequestered lysosomal pool through the coated-pit pathway (Table 2, Fig. 5). Independent measurements of the amount of cholesterol moving through HDL to the liver is nearly the same in the control (100 mg/day per kg) and mutant mice (108 mg/day per kg) (Fig. 2), illustrating that this increase in synthesis in the extrahepatic organs does, indeed, fully compensate for the sterol functionally lost to sequestration. Once taken up into the liver of the NPC$^{-/-}$/LDLR$^{+/+}$ mice, the cholesterol apparently enters a metabolically active pool of sterol that can be secreted into the gastrointestinal tract (85 mg/day per kg) or converted to bile acids and then secreted (65 mg/day per kg) (Table 2, Fig. 5). This flow of cholesterol out of the liver and into the feces is fully accounted for by the amount of cholesterol brought to the liver from the extrahepatic compartment by HDL (108 mg/day per kg) and newly synthesized in the liver (51 mg/day per kg) (Table 2, Fig. 5). Thus, sterol reaching the liver through the HDL/SR-BI pathway or newly synthesized in the hepatocytes apparently is fully available in a metabolically active pool for further biochemical transformation or for secretion.

This concept that in NPC disease cholesterol entering cells through these two different pathways has different metabolic fates is further supported by the observations in the adrenal of these animals. In the rodent, this gland is known to express high amounts of both SR-BI and LDLR and, further, to take up cholesterol from HDL at rates that are about four times higher than from LDL (22, 39, 52, 56, 57). This finding is confirmed in this study where the rate of sterol uptake from HDL equaled 1.1 mg/day per kg but from LDL was only 0.25 mg/day per kg (Fig. 3A and C). Hence, the pool of excess unesterified cholesterol is only modestly elevated in this tissue and is nearly eliminated when LDLR activity is abrogated (Fig. 3B). In contrast, the high concentration of esterified cholesterol is uniquely maintained in the adrenal. Furthermore, this pool is unaffected by deletion of either NPC1 or LDLR activity (Fig. 3D), as is the concentration of circulating steroid hormones. Thus, in the adrenal, as in the liver, LDL-C entering through the coated-pit pathway apparently becomes

trapped in the lysosomal compartment while HDL-C entering through the SR-BI pathway is metabolized normally.

These studies also provide further evidence that the pool of unesterified cholesterol sequestered in lysosomes is virtually unavailable to the cells as a regulator of metabolic events or as a substrate for biochemical transformation or secretion into bile. In the liver, for example, cholesterol entering the lysosomal compartment in the NPC animals cannot effectively regulate the rate of sterol synthesis or increase the level of cholesterol 7α-hydroxylase activity through interaction with the LXRα nuclear receptor (Fig. 4) (17). Furthermore, this sterol apparently cannot reach the endoplasmic reticulum where it normally would be esterified or 7α-hydroxylated as the first step in the synthesis of bile acid (Table 3) (17). Finally, this sequestered cholesterol also cannot be secreted into the bile but, rather, apparently is trapped nearly irreversibly in the cell. Thus, as implied in Fig. 5, the mutation in NCP1 essentially isolates cholesterol flowing into the cell through the coated-pit pathway from that entering the cell through the SR-BI pathway or from de novo synthesis. This conclusion is remarkably similar to that reached in studies of human subjects with NPC disease. Using the technique of multicompartmental analysis, it has also been concluded that the movement of cholesterol derived from HDL and de novo synthesis is unperturbed by the NPC mutation, but there is a marked abnormality in the trafficking of sterol derived from LDL (58).

However, as also suggested in Fig. 5, in the normal animal these three sources of cellular cholesterol feed into a single, metabolically active pool in the liver that controls sterol metabolism in the cell and provides substrate for various biochemical and secretory pathways. Several lines of evidence support this conclusion. First, in the steady state where lipid secretion into bile is constant, the predominant source for biliary cholesterol can be shifted from HDL-C to LDL-C to newly synthesized sterol by various experimental manipulations (59–61). Second, under similar steady-state circumstances where newly synthesized cholesterol is the predominant source for biliary lipid, the specific activity of the cholesterol in bile is significantly higher than the specific activity of the pool of sterol in the whole liver or in the circulation. This finding indicates that newly synthesized cholesterol can enter the metabolically active pool directly and be used as a source for biliary cholesterol (60). Third, under non-steady-state conditions, overexpression of SR-BI leads to increased clearance of HDL-C into the liver and an increase in the appearance of cholesterol in bile (62). Similarly, biliary cholesterol secretion increases when LDLR activity is enhanced pharmacogically and LDL-C is administered intravenously to the experimental animal. Finally, when a rodent is chronically treated with an HMG-CoA reductase inhibitor and this inhibitor is then acutely withdrawn, there is a period of inappropriate overproduction of newly synthesized cholesterol in the liver, and this overproduction is associated with an increase in both the level of cholesteryl esters and rate of secretion of cholesterol into the bile (63). Taken together, these various observations strongly suggest that in the normal animal, cholesterol that is newly synthesized in the hepatocyte as well as sterol reaching the liver through both the coated-pit and SR-BI pathways all feed into a single, metabolically active pool that functions to maintain cholesterol homeostasis in the liver cell.

Finally, while these studies have defined in quantitative terms the flow of cholesterol through the tissues of the NPC mouse, they have also raised three important new issues in which further studies are warranted. First, these experiments suggest that the severity of the liver disorder seen in NPC disease may be related to the amount of cholesterol reaching this organ from the diet. If this is, in fact, the case, then it would provide a possible therapeutic approach to ameliorating the liver dysfunction seen in the newborn child with NPC disease. Second, cholesterol accumulation in the neurons of the central nervous system and the role of this accumulation in nerve cell death has yet to be established. Such information is critical, however, for understanding the neurological dysfunction and dementia that is also an important part of the NPC syndrome. Finally, while the details of lipoprotein processing through the coated-pit pathway are fairly well understood, there is currently little information on the cell biology of how cholesteryl esters selectively taken up through the SR-BI pathway are processed and eventually reach the metabolically active pool of cholesterol in the cell. Clearly, answers to these questions are critically important for better understanding cholesterol processing in cells and for designing possible therapeutic manipulations for treating NPC disease.

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