Cholesterol substrate pools and steroid hormone levels are normal in the face of mutational inactivation of NPC1 protein

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Abstract Mutational inactivation of NPC1 largely blocks the movement of LDL-derived cholesterol from the lysosome to the metabolically active, cytosolic pool of sterol that is the substrate for steroid hormone production. Such a block might, in theory, lead to deficiencies in circulating levels of testosterone, progesterone, and corticosterone. However, there are at least two other sources for cellular cholesterol, de novo synthesis and scavenger receptor class B type I-mediated uptake of HDL cholesteryl ester (CE). In this study, we measured the rates of net cholesterol acquisition by these three pathways in the adrenal, ovary, and testis. In all three organs, the majority (81–98%) of cholesterol acquisition came from the selective uptake of CE from HDL and de novo synthesis. Furthermore, in the $npc1^{-/-}$ mouse, the cytosolic storage pool of CE in a tissue such as the adrenal remained constant ($\sim$25 mg/g). As a result of these alternative pathways, the plasma concentrations of testosterone (3.5 vs. 2.5 ng/ml), progesterone (8.5 vs. 6.7 ng/ml), and corticosterone (391 vs. 134 ng/ml) were either the same or elevated in the $npc1^{-/-}$ mouse, compared with the control animal. Thus, impairment of cholesterol acquisition through the NPC1-dependent, clathrin-coated pit pathway did not limit the availability of cholesterol substrate for steroid hormone synthesis in the steroidogenic cells.—Xie, C., J. A. Richardson, S. D. Turley, and J. M. Dietschy. **Cholesterol substrate pools and steroid hormone levels are normal in the face of mutational inactivation of NPC1 protein.** *J. Lipid Res.* 2006. **47:** 953–963.

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Essentially all cells acquire cholesterol primarily through de novo synthesis (1–3). In addition, at least three different proteins located in the plasma membrane are recognized that can also bring about the net transfer of sterol from the extracellular environment into the metabolically active pools of cholesterol within these cells. The first of these,

the LDL receptor (LDLR), can take up lipoproteins that contain one of two ligands, apolipoprotein B_{100} (apoB$_{100}$) or apoE, and these include LDL and the remnants of both VLDL and chylomicrons (4, 5). This transport process involves binding of the ligand to the receptor, clustering of the bound lipoproteins into clathrin-coated pits, and finally, internalization of these particles into the endosomal/lysosomal compartment of the cell (6). There, acidification leads to a change in the configuration of the binding site on the LDLR, release of the lipoprotein, and ultimately, hydrolysis of the cholesteryl ester (CE) contained in these particles by an acidic cholesteryl ester esterase, i.e., acid lipase (7–9). Thus, this LDLR-dependent endocytosis results in the uptake of the cholesterol, both esterified and unesterified, carried in particles like LDL, and deposits it as unesterified sterol in the endosomal compartment of the cell.

The second plasma membrane protein, scavenger receptor class B type I (SR-BI), binds lipoproteins containing apoAI such as HDL, and then processes these particles through a very different pathway (10, 11). Although the details of this internalization step are not clear, in some manner cholesteryl ester from the HDL particle is selectively translocated into the cell while the protein component of the lipoprotein is left in the extracellular fluid to be either reutilized or degraded in the kidney (12–14). This cholesteryl ester is then hydrolyzed by a neutral cholesteryl ester esterase, and the unesterified sterol is delivered directly into the metabolically active pools in the cell (15, 16). Importantly, this intracellular pathway bypasses the acidification step involved in the LDLR-dependent, clathrin-coated pit pathway. Thus, mutation of the acidic cholesteryl ester esterase (as seen in Wolman's disease) leads to a defect in the hydrolysis of esters arriving at the cell in

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Abbreviations: apolipoprotein B_{100}, apoB$_{100}$; CE, cholesteryl ester; LDLR, LDL receptor; NPC, Niemann-Pick disease type C; NPCIL1, Niemann-Pick type C1 Like 1; NPC1, Niemann-Pick type C1; SR-BI, scavenger receptor class B type I; TC, total cholesterol.

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LDL, but has no effect on the hydrolysis of cholesteryl esters delivered to the cell in HDL (17).

The third protein found in the plasma membrane that translocates cholesterol manifests a still different set of characteristics. Niemann Pick type C1 Like 1 (NPC1L1) protein appears to be able to move unesterified cholesterol from a micellar solution across the cell membrane directly to the intracellular metabolically active pools (18–20). This pathway does not require the intervention of a cholesteryl ester esterase. Importantly, NPC1L1 is expressed, and functions, primarily in the brush border of the intestinal epithelial cell (19). In contrast, both SR-BI and LDLR function primarily in the liver and steroidogenic tissues, although low levels of LDLR-dependent transport are found in many other cell types, including glia and neurons of the central nervous system (21, 22).

Although not expressed in the plasma membrane, another sterol transporter, known as Niemann Pick type C1 (NPC1) protein, that is related to NPC1L1, is located in the membranes of the endosomal/lysosomal complex and is also involved in the translocation of unesterified cholesterol (23, 24). This protein functions in tandem with the LDLR to facilitate the movement of unesterified sterol from the endosomal/lysosomal complex to the metabolically active pool of cholesterol in cells. When this protein is mutated, as in the syndrome known as Niemann Pick type C (NPC) disease, LDLR-dependent endocytosis takes place normally, but the unesterified cholesterol that is generated becomes irreversibly trapped in the late endosomal/lysosomal compartment of cells and so cannot reach the metabolically active pool in the cytosol (25–27). As a result, the content of unesterified cholesterol increases in every organ, including such diverse tissues as muscle, liver, the endocrine organs, and cells of the central nervous system (28–30). This accumulation is associated with cell death in organs such as the liver and in the central nervous system and, in both the mouse and human, results in significant liver and central nervous system disease (31–34).

It is possible that steroid hormone production might be compromised in NPC disease if the block in LDLR-dependent cholesterol uptake limited the availability of cholesterol to act as substrate for the synthesis of hormones such as testosterone, progesterone, and corticosterone. Indeed, the plasma testosterone concentration, as well as the weight of the seminal vesicles, has been reported to be significantly lower in older homozygous NPC mice, compared with control animals (35). Importantly, it has also been found that the synthesis of neurosteroids such as 5α-dihydroprogesterone and allopregnanolone by glial cells and neurons in the central nervous system is lower in NPC disease (36). If there is widespread deficiency of steroid hormone production in the NPC animal (and child), this deficiency might play a role in explaining some of the pathological features seen in both the liver and central nervous system in this syndrome.

These studies, therefore, were undertaken to quantify the three pathways by which the steroidogenic tissues acquire cholesterol, and to determine if there is limitation of substrate availibility that might lead to hormone de-

ficiency in the NPC syndrome. Initial studies measured the rates of clearance of cholesterol carried in both HDL and LDL into the adrenal, ovary, and testis. Synthesis rates were next measured in these same tissues. From these two sets of data, the absolute magnitude of cholesterol acquisition through the SR-BI- and LDLR-dependent transport pathways and from de novo synthesis was calculated. These pathways, as well as their respective contributions to the cholesteryl ester pool used for hormone synthesis, were then quantified in mice homozygous for a mutation in the NPC1 protein. Finally, plasma concentrations of testosterone, progesterone, and corticosterone were measured in both control and NPC mice. These studies demonstrate that cholesterol carried in LDL and taken up into the cells through the clathrin-coated pit pathway makes only a minor contribution to the cytosolic pool of sterol used for hormone synthesis in all three of these endocrine tissues. As a result, the plasma levels of testosterone, progesterone, and corticosterone are not reduced in the face of a mutation in the NPC1 protein.

MATERIALS AND METHODS

Animals and diets

These experiments utilized four groups of genetically modified mice. These groups included animals lacking functional LDL receptor activity (designated as $ldlr^{-/-}$) and mice lacking scavenger receptor class B type I activity ($srb1^{-/-}$) (2, 10, 37, 38). Mice homozygous for a mutational inactivation of the Niemann Pick type C1 protein, designated $npc1^{-/-}$, were utilized either in studies designed to measure cholesterol transport or hormone levels or to create double genetic deletions designated $npc1^{-/-}/ldlr^{-/-}$ (24, 28, 39). Similar groups of animals were used as the appropriate control mice in specific experiments and are designated $ldlr^{+/+}$, $srb1^{+/+}$, and $npc1^{+/+}$ in the various figures. These animals were housed in plastic colony cages in rooms with alternating 12 h periods of light and dark. After weaning and genotyping by the end of the third week, all animals were fed ad libitum a low-cholesterol (0.02%, w/w) pelleted diet (No. 7001 Harlan Teklad; Madison, WI) until they were studied at about 49 days of age. All studies were carried out during the fed state 1 h before the end of the dark cycle, and the experimental groups contained equal numbers of male and female animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Isolation and radiolabeling of mouse LDL and HDL

Mouse plasma was harvested from both male and female $ldlr^{-/-}$ mice maintained on the low-cholesterol diet. The LDL and HDL fractions were isolated by preparative ultracentrifugation in the density range of 1.020–1.055 g/ml and 1.063–1.21 g/ml, respectively. The LDL was then radiolabeled with either [^{125}I]tyramine cellobiose (TCB) or ^{131}I (2, 12, 39, 40). The apoE-containing HDL contaminating this LDL fraction was removed by passing the lipoprotein solution over a heparin-Sepharose Cl-6b column (41). After dialysis, these radiolabeled preparations were passed through a 0.45 μm Millex-HA filter immediately prior to injection into the recipient animals. The HDL was labeled with either [1α, 2α (n)-^{3}H]cholesteryl oleyl ether or [cholesteryl-4-^{14}C] oleate by exchange from donor liposomes (42, 43). Freshly

collected plasma from cholesteryl ester transfer protein (CETP) transgenic mice was used as the source of CETP (44). The labeled HDL was re-isolated by ultracentrifugation and dialyzed against saline. All four radiolabeled preparations of LDL and HDL were used within 48 h of preparation.

Measurement of LDL and HDL cholesterol clearance rates in vivo

Mice were anesthetized and a catheter was inserted into the jugular vein. After awakening, each animal was given a bolus of [^{125}I]TCB-labeled LDL followed by a continuous infusion of the same preparation at a rate determined to maintain a constant specific activity in the plasma (45). Ten minutes before the termination of this continuous 4 h infusion, a bolus of ^{131}I-labeled LDL was administered to each of the animals. The animals were then exsanguinated at 4 h, and nine different organs were removed. The remaining carcass was homogenized. Tissue and plasma samples were assayed for their content of ^{125}I and ^{131}I (2, 45, 46). A similar procedure was used for measuring HDL cholesteryl ester clearance rates. Animals were administered a priming dose of the [^{3}H]cholesteryl oleyl ether-labeled HDL followed by a continuous infusion of the same radiolabeled lipoprotein for 4 h. Ten minutes before termination of this infusion, the animals were given an intravenous injection of the [^{14}C]cholesteryl oleate-labeled HDL. The various organs, remaining carcass, and plasma samples were saponified in alcoholic KOH, and sterols were extracted and assayed for their ^{3}H and ^{14}C content. In both cases, the isotope injected 10 min before termination of the 4 h infusion was used to correct each tissue for contaminating plasma. The rates of clearance of the LDL and HDL particles by each of the tissues were then calculated and were expressed as microliters of plasma cleared of LDL or HDL cholesterol per hour per gram wet weight of each organ (μl/h/g). It should be noted that because of the difference in the labeling technique for these two particles, these rates represent the clearance of total cholesterol (TC) carried in the LDL particle that enters the cells through the clathrin-coated pit pathway but the clearance of only CE carried in HDL and entering the cells through the SR-BI pathway. Hence, in the various figures, these rates are designated as the clearance of LDL-TC and HDL-CE, respectively.

Measurement of rates of cholesterol synthesis in vivo

Each animal was administered 50–100 mCi of [^{3}H]water intraperitoneally. One hour later, the animals were anesthetized, blood was aspirated from the inferior vena cava, and the organs were removed and saponified (2, 47). After isolation of the tissue sterols and quantitation of their ^{3}H content, the rates of cholesterol synthesis in each organ were determined and expressed as nanomoles of [^{3}H]water incorporated into digitonin-precipitable sterols per hour per gram wet weight of tissue (nmol/h/g). Because the ^{3}H:C incorporation ratio is known (48), these incorporation rates could be converted to absolute rates of cholesterol synthesis. These latter rates were expressed as the micrograms of cholesterol synthesized per hour per gram of tissue (μg/h/g).

Measurement of plasma and tissue cholesterol concentrations

The plasma total cholesterol concentration was measured enzymatically (Kit No. 1127771; Boehringer Mannheim, Indianapolis, IN). Tissues were removed from the animals at the end of the experiments and extracted in chloroform-methanol (2:1; v/v), and the unesterified and esterified cholesterol was separated on Sep-Pak Vac RC cartridges (Waters; Milford, MA). The cholesterol in each fraction was then quantitated by gas-liquid chromatography (46, 49). These values were presented as milligrams of unesterified and esterified cholesterol per gram wet weight of tissue (mg/g).

Histological examination of the endocrine tissues

Testes, ovaries, and adrenals were harvested, fixed in 10% buffered formalin, and embedded in paraffin. These blocks were then sectioned (5 μm thick) and stained with hematoxylin and eosin.

Measurement of plasma hormone levels

Measurements of plasma testosterone, progesterone, and corticosterone levels were carried out by a commercial laboratory (Oregon Regional Primate Research Center, Beaverton, OR). The plasma for these measurements was obtained at the time of the studies 1 h prior to the end of the dark phase. Because of the documented variation in some of these hormone levels (50, 51), there were from 12 to 24 animals in each of the eight groups of mice that were studied.

Calculations

The data from all experiments are represented in the figures and in the table as the mean $\pm$ 1 SEM. The two-tailed unpaired Student's *t*-test was used to compare the various sets of data. Where appropriate, the significance of differences was tested at the $P < 0.05$ level.

RESULTS

Rates of SR-BI-dependent and LDLR-dependent lipoprotein clearance

As has been reported, there are high levels of expression of the mRNAs for both SR-BI and LDLR in the liver and various steroidogenic organs of the mouse. However, such expression does not necessarily indicate that there is significant net cholesterol uptake through these pathways in these various organs. The first study, therefore, quantified the rates of clearance of CE from HDL into these tissues that could be attributed to the activity of SR-BI. In control $srb1^{+/+}$ mice, the plasma HDL-CE concentration equaled 65 $\pm$ 3 mg/dl, and this CE was cleared by the whole animal at a rate of 95 $\pm$ 4 μl/h. As shown in **Fig. 1A**, the rates of CE clearance by the liver, adrenal, ovary, and testis, respectively, equaled 53, 172, 10, and 3 μl/h per gram tissue. As is also apparent, the other organs, including the residual tissues of the carcass, also demonstrated low rates of clearance. In the $srb1^{-/-}$ animals lacking SR-BI function, the plasma HDL-CE concentration was increased to 182 $\pm$ 10 mg/dl, and this CE was cleared by the whole animal at a rate of only 25 $\pm$ 3 μl/h. However, there was proportionately an even greater decline in lipoprotein cholesterol uptake in the liver, adrenal, ovary, and testis, which cleared the CE at only 8, 13, 2 and 1 μl/h/g, respectively (Fig. 1A). These clearance values in the $srb1^{-/-}$ mice were subtracted from those in the control $srb1^{+/+}$ animals in order to calculate the SR-BI-dependent component of HDL-CE clearance in each organ. As seen in Fig. 1B, only three tissues manifested significant SR-BI-dependent HDL-CE clearance, and these included the liver, adrenal, and ovary. The residual clearance values found in the testis
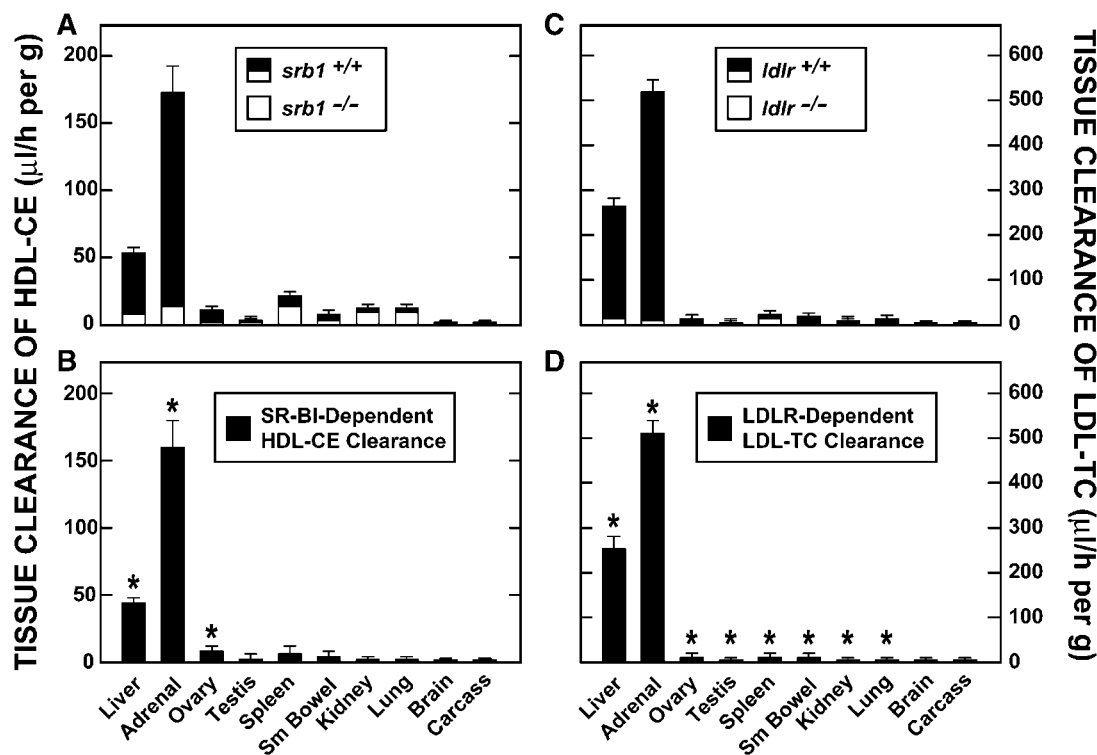
Fig. 1. Rates of clearance of HDL-cholesteryl ester (HDL-CE) and LDL-total cholesterol (LDL-TC) in 10 tissues of the mouse. The selective uptake of cholesteryl ester from HDL was measured in both 49-day-old control mice and in animals lacking scavenger receptor class B type I (SR-BI) activity (A). These latter rates of clearance found in the $srb1^{-/-}$ animals were subtracted from the rates determined in the $srb1^{+/+}$ mice to quantify the SR-BI-dependent clearance of cholesteryl ester in each of these organs (B). Similarly, the rates of clearance of TC from LDL were measured in 49-day-old $ldlr^{+/+}$ and $ldlr^{-/-}$ mice (C). The rates of clearance found in the $ldlr^{-/-}$ mice were subtracted from the rates determined in the $ldlr^{+/+}$ animals to obtain the rates of LDLR receptor (LDLR)-dependent TC uptake into each of these organs (D). Mean values ± 1 SEM are shown for 6–8 animals in each group. * in B, D, values significantly different from zero.

and remaining organs, including the brain and carcass, were not significantly different from zero.

Similar measurements were next made to determine the magnitude of TC uptake from LDL by these same organs that was dependent upon the presence of the LDLR. In the control $ldlr^{+/+}$ mice, the plasma LDL-TC concentration was 7 ± 1 mg/dl, and this TC was cleared by the whole animal at a rate of 550 ± 37 μl/h. As shown in Fig. 1C, the highest transport activity was found in the liver and adrenal, which cleared LDL-TC at rates of 265 and 517 μl/h/g, respectively. Much lower rates of clearance were found in the remaining organs, including the brain and carcass. As has been previously reported (2), these clearance rates were markedly lower in the $ldlr^{-/-}$ mice, where the LDL-TC concentration was 122 ± 8 mg/dl, and this TC was cleared by the whole animal at a rate of only 63 ± 9 μl/h. As seen in Fig. 1C, clearance of LDL-TC in the liver and adrenal of these animals was also reduced markedly to 15 and 10 μl/h/g, respectively. These clearance rates in the $ldlr^{-/-}$ mice were subtracted from the values found in the $ldlr^{+/+}$ animals to yield the values for the LDLR-dependent TC uptake depicted in Fig. 1D. In this case, rates of LDLR-dependent TC uptake significantly greater than zero were detected in eight different organs, although the highest values obviously were seen in the liver and adrenal. No LDLR-dependent TC uptake was detected in the brain or

tissues of the residual carcass, however. As an aside, whole-animal LDL-TC clearance was normal (510 ± 48 μl/h) in the $srb1^{-/-}$ animals, as was the clearance of HDL-CE (89 ± 5 μl/h) in the $ldlr^{-/-}$ mice.

Rates of cholesterol synthesis in the endocrine tissues

Apart from this uptake of cholesterol carried in HDL or LDL, each of these steroidogenic tissues also is capable of de novo sterol synthesis. In order to quantitate the magnitude of this biosynthetic pathway, rates of cholesterol synthesis were next measured in a parallel group of nine 49-day-old mice. The rates of incorporation of [³H]water into sterols were highest in the adrenal (1,479 ± 155 nmol/h/g) and ovary (237 ± 32 nmol/h/g) but relatively low in the testis (90 ± 3 nmol/h/g).

Rates of cholesterol acquisition in the endocrine tissues of normal mice

From these rates of [³H]water incorporation into cholesterol and the rates of SR-BI-(Fig. 1B) and LDLR (Fig. 1D) -dependent lipoprotein cholesterol uptake, it was possible to calculate the absolute magnitude of cholesterol acquisition through these three different mechanisms in the major steroidogenic tissues of the mouse. The results of these calculations are shown in **Table 1**. As is

TABLE 1. Rates of cholesterol acquisition from de novo synthesis and from HDL-CE and LDL-TC uptake in the adrenal, ovary, and testis

| | Cholesterol Acquisition from– | | |
	Synthesis	HDL-CE	LDL-TC
	$\mu g/h/g$		
Adrenal	46	104	35
Ovary	7	5.2	0.8
Testis	3	1.3	0.1

HDL-CE, HDL-cholesteryl ester; LDL-TC, LDL total cholesterol. These data were calculated from measurements made in 49-day-old control ($srb1^{+/+}/ldlr^{+/+}$) mice and represent the mass of cholesterol (μg) acquired either from de novo synthesis or from the uptake of HDL-CE or LDL-TC by the adrenal, ovary, and testis. The rates of acquisition from synthesis were calculated from rates of incorporation of [^{3}H]water into sterols by these three endocrine tissues. The rates of acquisition from HDL-CE and LDL-TC were calculated as the product of the respective clearance rates for each of these particles (Fig. 1) and their respective mean concentrations in the plasma of these same animals.

apparent, cholesterol acquisition by the uptake of LDL-TC through the clathrin-coated pit pathway made only a minor contribution to the substrate pools of sterol in these three endocrine tissues. This contribution to total sterol acquisition apparently varied from only 2% in the testis to 19% in the adrenal. In contrast, the great majority of the sterol utilized by these three tissues came from SR-BI-dependent HDL-CE uptake and de novo sterol synthesis.

These quantitative measurements were also supported by the changes in tissue total sterol pools that were observed when each of these pathways was independently manipulated. For example, deleting SR-BI activity in the $srb1^{-/-}$ animals led to a marked decrease in the steady-state tissue cholesterol concentration in the adrenal (3.9 ± 0.1 vs. 26.9 ± 3.6 mg/g), ovary (1.9 ± 0.2 vs. 2.6 ± 0.4 mg/g), and testis (2.0 ± 0.1 vs. 2.4 ± 0.1 mg/g) as well as to an increase in the rate of sterol synthesis. In contrast, deletion of LDLR activity in the $ldlr^{-/-}$ mice led to essentially no change in steady-state tissue cholesterol concentrations and to only a minor increase in the rate of sterol synthesis in these tissues (2). Thus, in the adrenal, ovary, and testis of the normal animal, the great majority of cholesterol required for hormone synthesis must come from the uptake of HDL-CE and de novo synthesis, two sources of sterol that bypass the LDLR-dependent, clathrin-coated pit pathway.

Cholesterol acquisition and pools of unesterified and esterified cholesterol in the endocrine tissues of the $npc1^{-/-}$ mouse

Before exploring the contribution of each of these pathways to the sterol pools in the endocrine glands of the mouse with mutational inactivation of the NPC1 protein, it was first necessary to establish that this mutation did not alter either the LDLR- or SR-BI-dependent uptake pathways. This proved to be the case, in that the whole-animal clearance of LDL-TC was the same in the $npc1^{-/-}$ mouse (566 ± 25 μl/h) as in the control $npc1^{+/+}$ animals (550 ± 37 μl/h). Similarly, the transfer of CE from HDL into the various tissues took place at essentially equal rates in the normal (95 ± 4 μl/h) and $npc1^{-/-}$ (88 ± 5 μl/h) mouse. This was also true for the SR-BI- and LDLR-dependent

pathways in the endocrine tissues. Clearly, inactivation of NPC1 that moves unesterified cholesterol from the late endosomal/lysosomal compartment to the cytosol did not affect the rate of uptake of either HDL-CE or LDL-TC across the cellular plasma membranes. Sequestration of the LDL-TC in the late endosomal/lysosomal compartment of every tissue, however, was perceived by the cells as a net loss of sterol, and this resulted in a compensatory increase in cholesterol synthesis in the whole animals from 152 nmol/h/g to 226 nmol/h/g. This change represented an increase in the absolute rate of sterol synthesis from 114 mg of cholesterol per day per kilogram of body weight in the $npc1^{+/+}$ mice to 169 mg/d/kg in the mutant $npc1^{-/-}$ animals.

As a result of these changes, there was accumulation of unesterified cholesterol in these steroidogenic cells and the appearance of a fine vesicular infiltrate in the cytosolic compartment that has been described in other cell types in the NPC mouse (34, 52). As seen in **Fig. 2**, for example, the histological alterations seen in the testis were limited to the interstitial (Leidig) cells. In the $npc1^{+/+}$ animals (Fig. 2A), these cells uniformly stained eosinophilic, whereas in the $npc1^{-/-}$ mice, many of the Leidig cells were enlarged and stained only faintly because of fine vacuolization of the cytoplasm (Fig. 2B). Spermatogenesis appeared to be unaffected. Similarly, in the ovaries, there were striking abnormalities, in that atretic follicles were surrounded by large numbers of vacuolated cells rather than by degenerating, apoptotic granulosa and thecal cells (Fig. 2D). Islands of swollen, vacuolated cells persisted in the interstitium as well. In contrast to these changes, there were no obvious histological differences between the cells of the adrenal in the $npc1^{+/+}$ and those in $npc1^{-/-}$ animals (Fig. 2E, F).

From these various findings, it was evident that the interaction of the three pathways for cholesterol acquisition could only be explored quantitatively in the adrenal. In this tissue, the majority of the cells have endocrine function (Fig. 2E), whereas in the ovary and, particularly, testis, only a small percent of the cells in the whole organs are devoted to the synthesis of steroid hormones (53, 54). Hence, the changes in the cholesteryl ester pools and in the various transport pathways (Fig. 1, Table 1) are small in the ovary and testis, compared with the adrenal. The next experiment, therefore, measured the pools of unesterified and esterified cholesterol in the adrenal under conditions in which the activity of the SR-BI, LDLR, and NPC1 proteins was systematically deleted.

As shown in **Fig. 3**, in the control mice with all three pathways intact, the pool of unesterified cholesterol present predominantly in cell membranes equaled 4.5 ± 0.2 mg/g, whereas the large storage pool of cholesteryl esters in the cytosol equaled 29.5 ± 5.1 mg/g. However, with deletion of the SR-BI pathway in the $npc1^{+/+}/srb1^{-/-}/ldlr^{+/+}$ mice, this storage pool could not be maintained (0.3 ± 0.1 mg/g), although the membrane pool of unesterified cholesterol remained essentially constant (4.7 ± 0.3 mg/g). In contrast, in the $npc1^{+/+}/srb1^{+/+}/ldlr^{-/-}$ animals, there was no alteration of either the esterified (26.2 ± 7.0 mg/g) or unesterified (4.0 ± 0.5 mg/g) sterol pools. These findings
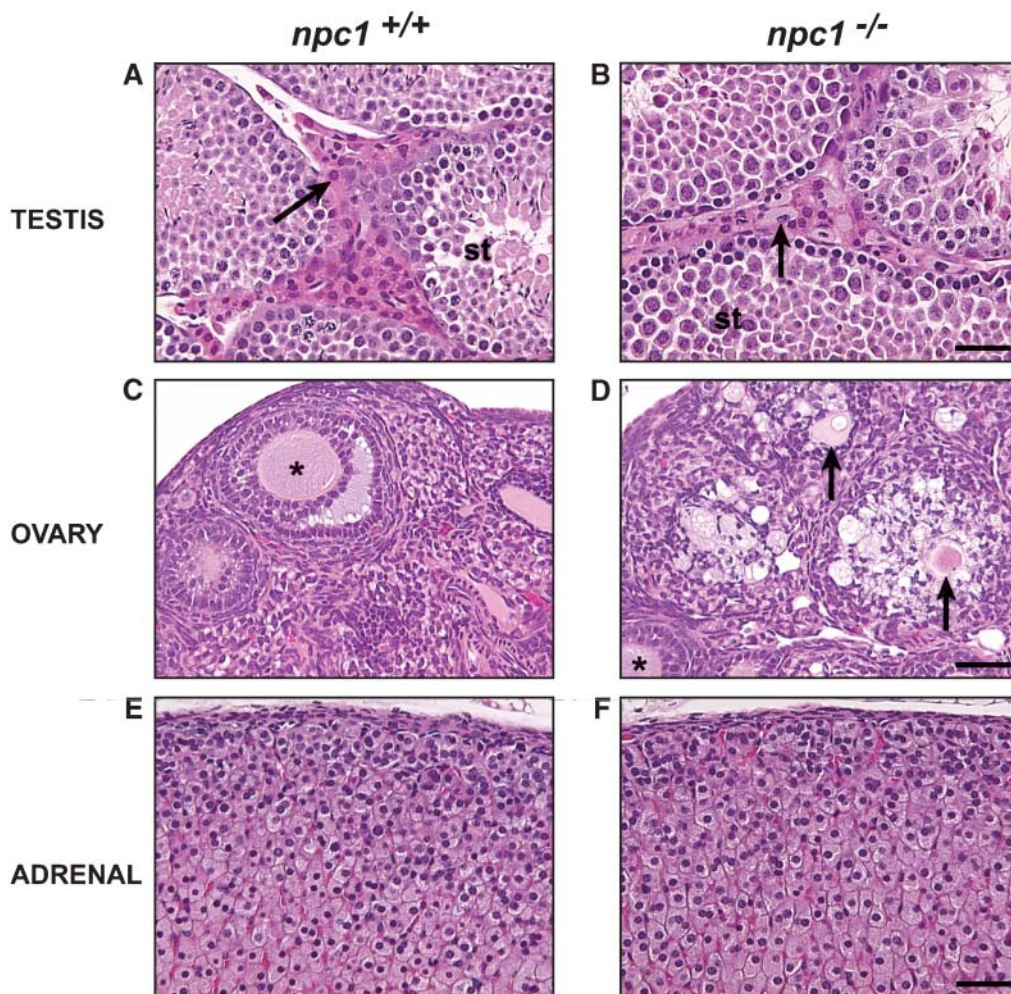
Fig. 2. Histologic sections of the testis, ovary, and adrenal from $npc1^{+/+}$ (A, C, E) and $npc1^{-/-}$ (B, D, F) mice. In the testis of the mutant male mouse (B), the interstitial (Leidig) cells contained finely vacuolated, pale-staining cytoplasm (arrow). In the ovary of the $npc1^{-/-}$ female (D), atretic follicles (arrows) were surrounded by large numbers of vacuolated granulosa cells. The cells of the adrenal cortex in the $npc1^{-/-}$ mouse (F) manifested no significant histological differences. st, seminiferous tubules; *, developing follicle; bars = 40 μm.

are all consistent with the earlier measurements of these pathways in the normal mouse.

When the NPC1 pathway was deleted while keeping both the SR-BI and LDLR pathways intact, as seen in the $npc1^{-/-}/srb1^{+/+}/ldlr^{+/+}$ animals, there was significant expansion of the total sterol pool in the adrenal (40.5 ± 6.2 mg/g) that resulted from a 3-fold increase of the unesterified cholesterol pool (14.5 ± 0.9 mg/g), whereas the cholesteryl ester pool remained unchanged (26.0 ± 6.2 mg/g). This expanded pool of unesterified cholesterol presumably was located in the cellular plasma membranes (~4 mg/g) and sequestered in the late endosomal/lysosomal compartment (~10 mg/g). To compensate for this sequestration, the rate of cholesterol synthesis in the adrenal increased 60% to 2,377 nmol/h/g. However, this expansion of the unesterified pool was largely prevented (7.0 ± 0.8 mg/g) when LDL-TC uptake through the clathrin-coated pit pathway was reduced in the $npc1^{-/-}/srb1^{+/+}/ldlr^{-/-}$ mice. The pool of esterified cholesterol, however, again

remained unchanged (25.5 ± 4.5 mg/g) in these animals lacking both NPC1- and LDLR-dependent transport activity.

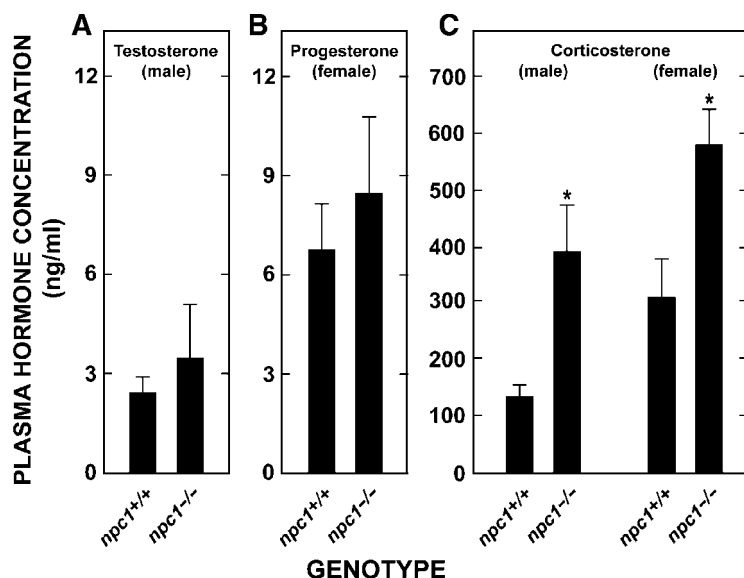### Plasma hormone levels in the $npc1^{-/-}$ mice

Taken together, these measurements provided strong direct evidence that the substrate for steroid hormone production in the adrenal, ovary, and testis of the mouse comes primarily from the uptake of HDL-CE and de novo synthesis and, therefore, should not be compromised by a mutation in NPC1. A final test of this hypothesis was carried out by measuring the levels of steroid hormones in a large number of normal $npc1^{+/+}$ and mutant $npc1^{-/-}$ animals, as shown in **Fig. 4**.

The concentration of testosterone in the plasma of mice is notoriously variable (50, 51). In these studies, for example, the range of values was very large in the $npc1^{+/+}$ mice (from 0.28 to 7.37 ng/ml). Nevertheless, there was no significant difference in the mean concentration of testosterone in the control (2.46 ± 0.42 ng/ml) and
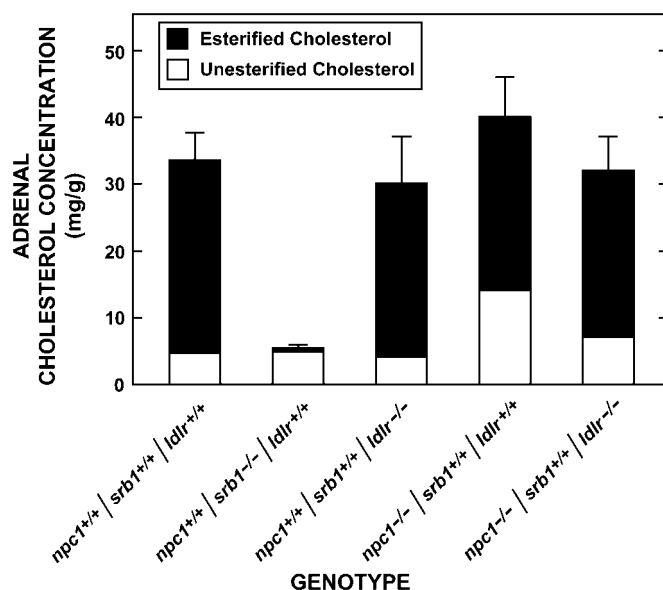
Fig. 3. Esterified and unesterified cholesterol concentrations in the adrenal gland of normal mice and in animals lacking SR-BI, LDLR, and Niemann-Pick disease type C1 activities. These animals were 49 days old. The adrenal glands were removed, the lipids were extracted, and the unesterified and esterified cholesterol was separated on columns and quantified. Mean values ± 1 SEM for 6–12 animals in each group are shown.

$npc1^{-/-}$ (3.46 ± 1.61 ng/ml) animals (Fig. 4A). Similar findings were observed with the levels of progesterone in the control (6.69 ± 1.41 ng/ml) and mutant (8.50 ± 2.21 ng/ml) mice (Fig. 4B). Corticosterone levels, in contrast, were actually significantly higher in the $npc1^{-/-}$ mice than in the $npc1^{+/+}$ animals (Fig. 4C). This was true in both the male and the female animals that, at this age, were symptomatic and possibly stressed by their underlying neurological disease. Thus, these results clearly confirmed that there was no limitation of available substrate for steroid hormone production in the adrenal, ovary, and
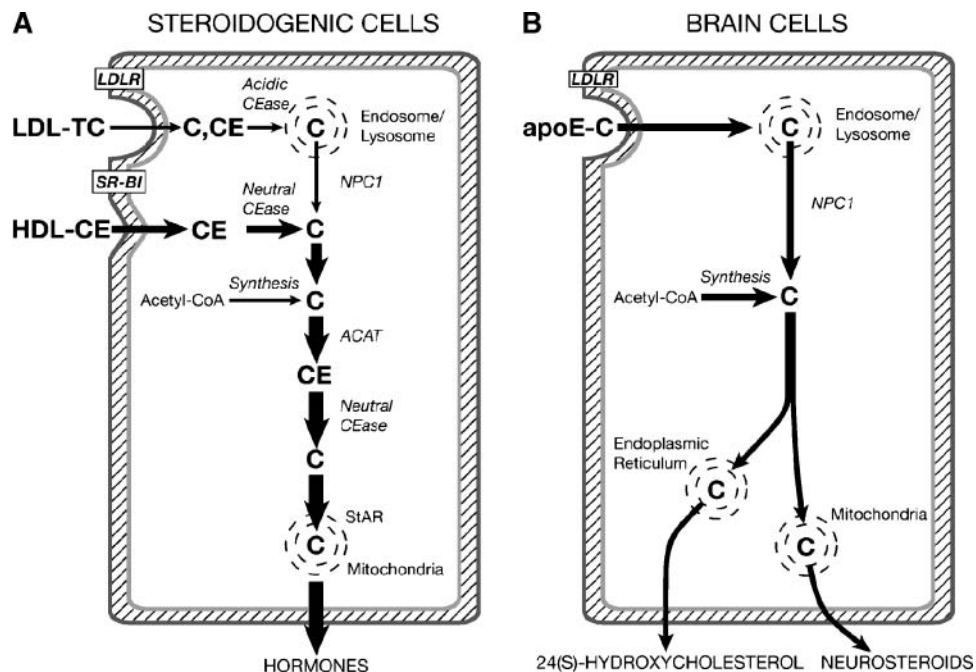
testis of the mouse with mutational inactivation of the NPC1 pathway.

DISCUSSION

These studies provide direct, quantitative measurements of the major pathways by which the steroidogenic tissues in the mouse acquire cholesterol for hormone synthesis. As illustrated in **Fig. 5A**, these pathways include the receptor-dependent transport of LDL-TC through the clathrin-coated pit system, selective uptake of HDL-CE utilizing SR-BI, and de novo synthesis. The cholesteryl ester contained in the LDL particle is hydrolyzed to unesterified cholesterol in the lysosomal compartment by a cholesteryl ester esterase with an acidic pH optimum, but the cholesteryl ester taken up through the SR-BI pathway is hydrolyzed in the cytosol by a neutral cholesteryl ester esterase. While mutational inactivitation of NPC1 leads to accumulation of unesterified cholesterol derived from LDL-TC in the endosomal/lysosomal compartment, the processes of cholesterol acquisition through the selective uptake of HDL-CE and de novo synthesis are intact, and supply fully adequate amounts of cholesterol for hormone synthesis in the adrenal, ovary, and testis.

The absolute rates of cholesterol acquisition through de novo synthesis and HDL-CE uptake account for 81–98% of the sterol required for steroid hormone synthesis in the normal mouse (Fig. 1, Table 1). This result is similar to those reported in other species (55). In the face of a mutation in NPC1, loss of the small contribution of sterol from the uptake of LDL-TC is readily compensated for by a modest increase in the rate of de novo synthesis in these cells. Consequently, in the adrenal, for example, the pool of cholesteryl ester used as substrate for hormone synthesis is unaffected by deletion of either LDLR or NPC1 activity (Fig. 3). In contrast, this pool cannot be maintained when the SR-BI transport system, the major source for cholesterol in this gland (Table 1), is lost (Fig. 3).

Fig. 4. Plasma steroid hormone levels in male and female control and $npc1^{-/-}$ mice at 49 days of age. The plasma from these animals was obtained 1 h before the end of the dark cycle. Testosterone and corticosterone levels were measured in male mice, and progesterone and corticosterone concentrations were measured in female animals. Mean values ± 1 SEM are shown for data obtained in 24, 20, 13, 12, 20, 18, 19, and 20 animals (left to right) in each group. The asterisks identify two values for the $npc1^{-/-}$ animals that were significantly higher than their respective $npc1^{+/+}$ controls.

Fig. 5. Diagrammatical representation of the major unidirectional pathways for cholesterol acquisition and disposition in steroidogenic (A) and brain (B) cells. Steroidogenic cells acquire cholesterol from at least three sources including LDL-TC, HDL-CE, and de novo synthesis, whereas cells in the central nervous system probably have only two sources, unesterified cholesterol complexed to apolipoprotein E and de novo synthesis. In the steroidogenic cells, the cholesterol is esterified by ACAT and temporarily stored in the cytosol until converted back to unesterified cholesterol, transported into the mitochondria, and metabolized to steroid hormones. In contrast, no cholesteryl esters can be identified in the brain, and the unesterified cholesterol is metabolized to either 24(S)-hydroxycholesterol in the endoplasmic reticulum or to neurosteroids in the mitochondria.

Because of these quantitative relationships, sufficient sterol substrate for hormone synthesis by the adrenal, ovary, and testis is available even when a mutation in NPC1 blocks cholesterol acquisition through the clathrin-coated pit pathway. Plasma testosterone, progesterone, and corticosterone levels are either normal or elevated in the $npc1^{-/-}$ mouse (Fig. 4).

These findings in the mouse are reflected in similar, although limited, observations in humans. In individuals who either lack LDLR function (homozygous familial hypercholesterolemia) or have little circulating LDL-TC (homozygous hypobetalipoproteinemia), the basal levels of circulating adrenal steroids are normal (56–58). Furthermore, the levels of these hormones also increase in such individuals in response to prolonged adrenocorticotropic hormone infusions, although these increases are not as great as seen in normal, control subjects. These results, therefore, suggest that in the human, as in the mouse, uptake of LDL-TC through the clathrin-coated pit pathway is not an important source of substrate for steroid hormone synthesis except, possibly, after prolonged stress. These findings are also consistent with the fact that we have been unable to locate any published reference to adrenal, ovarian, or testicular hormone deficiency in individuals with NPC disease.

These data for steroidogenic cells are similar to those previously described for other tissues and for the whole

animal. Unesterified cholesterol begins to collect in the tissues of the $npc1^{-/-}$ mouse in utero (52), and this sequestration continues daily until, at 7 weeks of age, the concentration of sterol is elevated in every organ, in general, and in the liver, in particular, because this latter organ is primarily responsible for clearing apoB$_{100}$- and apoE-containing lipoproteins from the plasma (28). Every cell readily responds to this slight deficiency of sterol brought on by sequestration of cholesterol entering the tissue through the clathrin-coated pit pathway by slightly increasing sterol synthesis. Because of this adaptation, there is probably no net deficit of cholesterol in the metabolic pool of any organ in the $npc1^{-/-}$ mouse. Hence, the level of cell membrane turnover, the concentrations of plasma testosterone, progesterone, and corticosterone, the rate of biliary cholesterol secretion, and the rate of bile acid synthesis are all essentially normal in the face of the NPC1 mutation (59). Nevertheless, this normalization of cholesterol fluxes in the $npc1^{-/-}$ animal comes as a result of an increased rate of cholesterol synthesis in the whole animal (182 mg/day per kilogram body weight compared with 120 mg/day/kg in control animals) that just offsets the continual functional loss of sterol (~60 mg/day/kg) through sequestration in the endosomal/lysosomal compartment of every cell (28). It is this adaptation in the rate of cholesterol synthesis that allows the cells of every organ to function essentially normally, so that the mouse or

human with the NPC mutation is able to survive and develop in utero and during early childhood.

This situation is very different from that reported in organisms such as *Drosophila* and *Caenorhabditis elegans*. For example, inactivation of the homolog of NPC1 in the fly does lead to deficient sterol substrate and diminished synthesis of the critical hormone ecdysone. Without this hormone, the larval form of *Drosophila* cannot molt (60, 61). However, unlike the mammal, these species have apparently lost the capacity to synthesize the sterol nucleus and so are cholesterol auxotrophs. Any mutation, therefore, that interferes with the processing of exogenous sterol would necessarily lead to a defect in hormone synthesis. The situation is very different in mammalian cells, all of which can apparently synthesize cholesterol and/or obtain it through pathways that bypass NPC1. Thus, the fact that inactivation of NPC1 in *Drosophila* or *C. elegans* leads to defective hormone synthesis has little relevance to the situation in steroidogenic cells in the mammal.

However, as also shown in Fig. 5, the situation in the central nervous system of the mammal could conceivably be different from that found in the other organs of the body. It is well known that plasma lipoproteins, including LDL, HDL, and the remnants of VLDL cannot cross the blood-brain barrier (22, 62, 63). Rather, during brain development, all of the cholesterol that is required for cell plasma membrane expansion and for myelination comes from the synthesis of sterol in both glial cells and neurons (22, 63). In addition, evidence from in vitro studies suggests that a portion of the cholesterol synthesized in astrocytes is complexed to apoE and carried to neurons, particularly during synapse formation (64–66). Thus, as summarized in Fig. 5B, neurons in the central nervous system may acquire cholesterol from both de novo synthesis and the uptake of an apoE-cholesterol complex utilizing the LDLR and clathrin-coated pit pathway. Not surprisingly, therefore, mutation of NPC1 results in the same lipid accumulation and vacuolization of the cytosol in brain cells as is seen in hepatocytes, Leidig cells, and ovarian cells (29, 34, 52).

A portion of this pool of unesterified cholesterol in the cells of the central nervous system is metabolized either in the endoplasmic reticulum to 24(S)-hydroxycholesterol and excreted from the brain (67–69) or in the mitochondria to various neurosteroids, such as 5α-dihydroprogesterone and allopregnanolone (36, 70, 71). With inactivation of NPC1 and accumulation of unesterified cholesterol in glial cells and neurons, there is diminished formation of both 24(S)-hydroxycholesterol and the neurosteroids (22, 69, 71). These lower rates of cholesterol metabolism through these two pathways are associated with decreased mRNA levels and enzymatic activities for the critical proteins CYP46A1, 3αHSD, CYP11A1, and SRD5A1 (36, 69, 72). Importantly, these metabolic pathways are expressed in glial cells and, particularly, in large metabolically active neurons such as Purkinje cells (67, 73). Such cells are among the first to degenerate in NPC disease (74, 75). Thus, the most likely explanation for these findings is that the diminished rate of formation of 24(S)-hydroxycholesterol and neurosteroids is the result of cell death and, as

in other organs, is not due to a shortage of sterol in the metabolically active substrate pools.

Taken together, these various observations suggest that the primary detrimental event in NPC disease is the accumulation of unesterified cholesterol and other lipids in the late endosomal/lysosomal compartment of cells throughout the body. Over time, this accumulation leads to cell death through apoptosis and inflammation in susceptible cell populations and, eventually, to clinical symptoms (34, 72). Thus, cholesterol accumulation in hepatocytes causes liver cell dysfunction and, eventually, cirrhosis (33, 76). Lipid accumulation in glial cells and neurons leads to similar glial and nerve cell death with the development of clinical symptoms (31, 32). Because cholesterol accumulates in all tissues throughout the body in this disorder, it is possible that further investigation will reveal more widespread organ dysfunction than is currently recognized. For example, plasma testosterone levels have been reported to be somewhat lower in mice older than those used in these studies (35). In any case, it is likely that effective treatment of NPC disease will involve manipulations that either lessen the uptake of lipoprotein cholesterol into cells though the clathrin-coated pit pathway or that overcome the defect in transfer of this sterol from the late endosomal/lysosomal compartment to the metabolically active pool of sterol in the cytosol.

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