Intracellular trafficking of the free cholesterol derived from LDL cholesteryl ester is defective in vivo in Niemann-Pick C disease: insights on normal metabolism of HDL and LDL gained from the NP-C mutation

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Abstract Niemann-Pick C disease (NP-C) is a rare inborn error of metabolism with hepatic involvement and neurological sequelae that usually manifest in childhood. Although in vitro studies have shown that the lysosomal distribution of LDL-derived cholesterol is defective in cultured cells of NP-C subjects, no unusual characteristics mark the plasma lipoprotein profiles. We set out to determine whether anomalies exist in vivo in the cellular distribution of newly synthesized, HDL-derived or LDL-derived cholesterol under physiologic conditions in NP-C subjects. Three affected and three normal male subjects were administered [^{14}C]mevalonate as a tracer of newly synthesized cholesterol and [^{3}H]cholesteryl linoleate in either HDL or LDL to trace the distribution of lipoprotein-derived free cholesterol. The rate of appearance of free [^{14}C]- and free [^{3}H]cholesterol in the plasma membrane was detected indirectly by monitoring their appearance in plasma and bile. The plasma disappearance of [^{3}H]cholesteryl linoleate was slightly faster in NP-C subjects regardless of its lipoprotein origin. Appearance of free [^{14}C]cholesterol in the plasma (and in bile) was essentially identical in normal and affected individuals as was the initial appearance of free [^{3}H]cholesterol derived from HDL, observed before extensive exchange occurred of the [^{3}H]cholesteryl linoleate among lipoproteins. In contrast, the rate of appearance of LDL-derived free [^{3}H]cholesterol in the plasma membrane of NP-C subjects, as detected in plasma and bile, was retarded to a similar extent that LDL cholesterol metabolism was defective in cultured fibroblasts of these affected subjects. These findings show that intracellular distribution of both newly synthesized and HDL-derived cholesterol are essentially unperturbed by the NP-C mutation, and therefore occur by lysosomal-independent paths. In contrast, in NP-C there is defective trafficking of LDL-derived cholesterol to the plasma membrane in vivo as well as in vitro. The in vivo assay of intracellular cholesterol distribution developed herein should prove useful to quickly evaluate therapeutic interventions for NP-C.—**Shamburek, R. D., P. G. Pentchev, L. A. Zech, J. Blanchette-Mackie, E. D. Carstea, J. M. VandenBroek, P. S. Cooper, E. B. Neufeld, R. D. Phair, H. B. Brewer, Jr., R. O. Brady, and C. C. Schwartz.** Intracellular trafficking of the free cholesterol derived from LDL cholesteryl ester is defective in vivo in Niemann-Pick C disease: insights on normal metabolism of HDL and LDL gained from the NP-C mutation. *J. Lipid. Res.* 1997. **38:** 2422–2435.

Supplementary key words cholesterol metabolism • intracellular transport • bile • kinetic model • lipoprotein

Niemann-Pick C disease (NP-C) is a rare, autosomal recessive, inborn error characterized by neonatal hepatitis and/or the late childhood appearance of a progressive spectrum of neurologic signs including vertical ophthalmoplegia, ataxia, dementia, and dystonia (1). The pathologic hallmark is lysosomal lipid storage primarily of free (unesterified) cholesterol and sphingolipids. Although historically linked to Niemann-Pick A and B diseases, NP-C has an unknown etiology, distinct from the primary lesions of sphingomyelinase of the former disorders. The majority of genotypically characterized NP-C cases have been linked to a mutation on chromosome 18q11.1 (2).

Abbreviations: NP-C, Niemann-Pick C disease; LDL, low density lipoprotein; HDL, high density lipoprotein; NSC, newly synthesized cholesterol.

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Cultured fibroblasts of individuals with NP-C display a unique defect of intracellular cholesterol trafficking. When the media of cholesterol-deprived NP-C fibroblasts is supplemented with LDL, the endocytosed LDL-cholesteryl ester is hydrolyzed but the free (unesterified) cholesterol product is then sequestered in lysosomes (1). This trapped cholesterol cannot rapidly distribute to the cell surface plasma membrane. The rate of enrichment of the plasma membrane has been reported to be one-third of normal in NP-C cells, causing delayed induction of the cell's homeostatic responses that are exquisitely sensitive to enrichment with cholesterol (3).

Communication between intracellular and extracellular cholesterol pools occurs at the plasma membrane. In most cells, over 95% of cholesterol is unesterified and greater than 80% resides in the plasma membrane (4). HDL and possibly other extracellular acceptors may dock on plasma membranes promoting rapid exchange and, in some circumstances, net transfer of cholesterol (5–7). The mechanisms by which intracellular organelles both enrich and deplete the plasma membrane are poorly understood. Enrichment of plasma membranes with cholesterol synthesized de novo in the endoplasmic reticulum is relatively rapid ($t^1/_2$ = 15 min) as is that derived from the hydrolysis of cytosolic cholesteryl ester droplets (8, 9). The rate of movement of lysosomally derived cholesterol has been reported to vary from 2 min (10) to several hours (11), a divergence likely reflecting both the experimental conditions and the level of lysosomal cholesterol loading. As this particular intracellular pathway has been shown to be deficient in cultured NP-C cells, it was of interest to determine whether the mutation might influence the transfer of cholesterol from intracellular to extracellular (plasma) pools in a physiologic in vivo setting. No overt abnormalities in the circulating lipoprotein profile have been reported in NP-C subjects, causing uncertainty with regard to the physiological consequences of the mutation on in vivo cholesterol metabolism. Monitoring the plasma appearance of [^{14}C]cholesterol formed de novo as well as [^{3}H]cholesterol derived from HDL and LDL cholesteryl ester has clearly documented a unique delay in the ability of NP-C subjects to mobilize their intracellular stores specifically from LDL-derived cholesterol.

METHODS

Subjects

Three males with NP-C and three normal males were studied in the Clinical Research Center at the Medical College of Virginia Hospital of VCU. Informed consent was obtained from all six volunteers and from a parent who accompanied each subject with NP-C. All subjects had normal renal, liver, and thyroid function tests, fasting blood glucose, and blood cell counts. For at least one month all subjects took a diet of 30–40% fat and 250–350 mg cholesterol per day. No subject took medication for at least one week before the study.

Each subject with NP-C had clinically normal growth and development until age 13–16 years when classic neurologic signs of NP-C appeared. Each had an older sibling with more advanced sequelae of NP-C and each was ambulatory, very active and either a part-time student or employee. NP-C #1 was age 19 years and had a weight of 75 kg, a height of 188 cm, and triglyceride of 187 mg/dl. Values for NP-C #2 were 39 years, 65 kg, 180 cm, and 92 mg/dl, respectively. Values for NP-C #3 were 20 years, 86 kg, 182 cm, and 170 mg/dl, respectively. NP-C #2 had a percutaneous endoscopic gastrostomy (PEG) tube placed 1 year previously for supplemental calories as needed because of intermittent swallowing difficulty.

Normal #1 was 55 years of age and had a weight of 84 kg, a height of 182 cm, and triglyceride of 149 mg/dl. The corresponding values for Normal #2 were 21 years, 70 kg, 170 cm, and 135 mg/dl, and for Normal #3; 20 years, 84 kg, 191 cm, and 74 mg/dl, respectively.

After overnight fasting, an enteral feeding tube (10–12 Fr) was passed via the nose or mouth except in NP-C #2 in whom it was passed via the lumen of the PEG tube. The opening was positioned in the mid duodenum with fluoroscopic guidance. Midazolam, 4 mg, was given by vein to NP-C #1 and #3 just before passing the enteral tube. Cholecystokinin octapeptide (Kinevac, Squibb Diagnostics, Princeton, NJ) was infused by vein at 1.0 mcg/h in each subject the entire time the enteral tube was in place to contract the gallbladder. About 10:30 AM (2.5 h after placing the tube) the two isotopic compounds were administered by vein over 2 min. Bile, 4 ml, was then collected at 20–40 min intervals from the duodenum with a syringe. The bile was immediately extracted in 20 volumes of chloroform–methanol 2:1 (vol:vol). Subjects took cholesterol-free clear liquids as desired while the tube was in place. After 8 h of bile collection the cholecystokinin infusion was stopped, the enteral tube was removed, and the usual diet was resumed. Blood was collected at various intervals as shown in the figures in tubes containing EDTA or heparin. The cells were immediately separated in a refrigerated centrifuge and the plasma was quickly extracted in chloroform–methanol, or ultracentrifuged prior to extraction.

Radiolabeled compounds

D,L[2-^{14}C]mevalonic acid and [1,2,6,7-^{3}H(N)]cholesteryl linoleate were obtained from NEN Research Prod-

ucts, Boston, MA, and stored at −15°C in ethanol and toluene, respectively. [^{14}C]mevalonic acid was prepared for administration in sterile saline as described previously (6). Normal #1 received 76 uCi of [^{14}C]mevalonic acid and the others received about 55 μCi. [^{3}H]cholesteryl linoleate was screened for purity by silicic acid column chromatography (12) and by thin-layer chromatography (13). The radiolabel was not used when purity was <93% by either method.

[^{3}H]cholesteryl linoleate was incorporated into each subjects' lipoproteins prior to administration by the following new method. One week before the study, after an overnight fast, 40 ml blood was collected in EDTA. Plasma was immediately separated in a refrigerated centrifuge. The plasma fraction of interest was subsequently maintained at 4–6°C under sterile conditions throughout preparation until 5 min before administration when warmed to 25°C. All dialyses were with sterile 0.9% NaCl, pH 7.4, 0.01% EDTA at 5°C. LDL (d 1.020–1.060 g/ml) was isolated from the plasma of Normal #2, NP-C #2, and NP-C #3 by sequential ultracentrifugation. A mixture of HDL and LDL (d 1.050–1.21 g/ml) was isolated in Normal #1 and NP-C #1; HDL (d 1.063–1.21 g/ml) was isolated in Normal #3. The lipoprotein in a volume of 7–8 ml was dialyzed overnight. Next, [^{3}H]cholesteryl linoleate, 100–200 μCi in 0.075 ml ethanol in a microliter pipette, was added dropwise over 3 min to the lipoprotein solution while gently shaking with interruptions every 45 sec for brief (1 sec) vortex. Sequential ultracentrifugation of the solution was then repeated at the original densities and the reisolated [^{3}H]lipoprotein was dialyzed overnight against four changes of the 1-liter baths. The autologous [^{3}H]lipoprotein was passed through 0.45 μm then 0.20 μm filters, an aliquot was saved for analysis, and the remainder was administered by vein within 1 h of filtration. Normal #1 received 67 μCi of [^{3}H]cholesteryl linoleate; NP-C #1 47 μCi; NP-C #2 40 μCi; Normal #2 and NP-C #3 27 μCi; and Normal #3 20 μCi.

About 80% of both phospholipid (14) and cholesterol masses in the initially isolated lipoprotein and about 30% of the added [^{3}H]cholesteryl linoleate were recovered in the final preparation. Agarose gel electrophoresis of each [^{3}H]LDL preparation revealed a single visible band that corresponded to LDL of whole plasma in the adjacent lane; two bands, corresponding to LDL and HDL, were seen in both [^{3}H]HDL/LDL preparations; and only HDL was seen in the [^{3}H]HDL preparation. The agarose gels were sliced and radioactivity was determined; 91% of ^{3}H was in the LDL band, 5% in the area corresponding to HDL, and the remainder was evenly spread out from the [^{3}H]LDL preparations; from the [^{3}H]HDL/LDL preparations 10–20% of ^{3}H was in the LDL band and the remainder in HDL; from the [^{3}H]HDL preparation, 97.3% of ^{3}H was in the HDL band. A volume of 0.01 ml from each ^{3}H-labeled preparation was mixed with 3.0 ml of the subjects' heparinized plasma at 5°C and heparin-manganese was added (15). In each [^{3}H]LDL preparation, 98% of the ^{3}H activity precipitated; 26–36% precipitated from the [^{3}H]HDL/LDL preparations, and 18% of the ^{3}H activity precipitated from the [^{3}H]HDL.

The location of ^{3}H in each administered preparation was analyzed. By HPLC, 91–95% was in cholesteryl linoleate and 2–3% was in other cholesteryl esters (mainly arachidonate). By silicic acid chromatography and precipitation with digitonide, we found that only 0.15% of administered ^{3}H activity for each subject was in free cholesterol; 2–5% was in more polar, unidentified sterols. The specific activity (normalized for weight and dose) of free cholesterol in the initial blood sample of each subject was 10–35 dpm/μmol, very close to that calculated from the amount of free [^{3}H]cholesterol administered; the value was subtracted from each plasma free cholesterol result.

Analytic procedures

Chloroform and methanol phases were separated by the addition of one-fifth volume of water (16). The chloroform phase was subjected to silicic acid column chromatography to separate free from esterified cholesterol (12). Free cholesterol mass was determined by gas–liquid chromatography and radioactivity (^{3}H and ^{14}C) as the digitonide as described previously (6). Cholesteryl heptadeconate (internal standard) was added to the esterified cholesterol sample. The sample was then suspended in isopropanol–acetonitrile 3:1 (v:v), warmed to 40°C, and vortexed just prior to HPLC using a 1 cm × 25 cm Ultrasphere ODS 5 μm column (Beckman Instruments) at 40°C, modified from the description of Carroll and Rudel (17). The mobile phase was acetonitrile–tetrahydrofuran–water 65:35:1 (v:v:v) pumped at 3.5 ml/min; detection was at 213 nm. The mass of each species was calculated using the internal standard method. The cholesteryl linoleate peak was collected and assayed for ^{3}H and ^{14}C activity. Identities of peaks were based on relative retention time of standards (cholesteryl esters of arachidonate, linoleate, stearate, palmitate, oleate, and 22:6; Sigma Chemical Co.).

The mass of each major bile acid was determined by injection of an aliquot of the methanol phase of bile on HPLC as modified from the description of Heuman et al. (18). Taurine and glycine conjugates were not separated. Progesterone was added to the aliquot and the mixture was suspended in mobile phase before injection on a 1 cm × 25 cm Ultrasphere ODS 5 μm column at 25°C; mobile phase was methanol–KH_2PO_4 0.03 M 75:25 (v:v), pH 6.5, pumped at 1.5 ml/min. Detection

was at a wavelength of 200 nm. Radioactivity in each bile acid was determined by scintillation counting of the individual peak collections (not done in Normal #3). Losses of ^{3}H from the 7-position of cholesterol and ^{14}C from the 26-position of cholesterol during bile acid synthesis were not corrected; both losses were assumed to be 15–20% of the radioactivity in cholesterol and to be the same for all subjects.

RESULTS

Plasma cholesterol was lower in NP-C than in normal subjects (**Table 1**). Of the plasma cholesteryl ester, Table 1, about 60% was comprised of cholesteryl linoleate in all subjects.

The in vivo human use of lipoproteins containing [^{3}H]cholesteryl linoleate tracer has not been described previously. **Figure 1** shows the disappearance of [^{3}H]cholesterol linoleate from plasma. The physiologic behavior of these ex vivo labeled autologous lipoproteins was supported by three in vivo findings. First the initial plasma sample, obtained within 25 min of ^{3}H administration and corrected for estimated total plasma volume (4.5% of body weight in kg) contained 93–104% of the ^{3}H injected, over 92% as [^{3}H]cholesteryl linoleate. Second, the sum of Y-intercepts ranged from 0.89 to 1.03 (Table 1). Taken together, the first and second findings show no evidence for rapid or unphysiologic removal of the ^{3}H tracer from plasma. Third, for 300 min after administration, the [^{3}H]cholesteryl linoleate gradually distributed among VLDL, IDL, LDL, and HDL in a physiologic fashion (**Table 2**).

[^{3}H]cholesteryl linoleate in autologous LDL (Normal #2, NP-C #2, and NP-C #3) or predominately (>78%) HDL (Normal #1 and #3, NP-C #1) was administered by vein. As the characteristics of free [^{3}H]cholesterol appearance in plasma and bile were the primary responses being monitored, it was essential to determine whether the initial disappearance rate of [^{3}H]cholesteryl lineolate from plasma varied among subjects or was influenced by injection in LDL versus HDL. This was important because rapid disappearance of [^{3}H]cholesteryl linoleate could initially contribute to rapid appearance and a larger quantity of free [^{3}H]cholesterol. The ^{3}H activity in plasma cholesteryl linoleate decreased gradually throughout the 3000–5000 min duration of each study (Fig. 1). Two exponentials fit each subjects [^{3}H]cholesteryl linoleate data except for NP-C #1 in whom the second was poorly defined probably related to the short duration (3000 min) of his study. The exponentials are shown in Table 1. Among the three NP-C subjects the first exponential, λ1, was remarkably similar. However, λ1 was about 35% higher in NP-C than in normal subjects so that free [^{3}H]cholesterol might be anticipated to appear faster in NP-C plasma and bile. Administration in LDL versus HDL did not seem to influence the disappearance of [^{3}H]cholesteryl linoleate from plasma probably because of relatively rapid exchange between lipoproteins (Table 2 and see below).

As vividly illustrated in **Fig. 2**, the rapid disappearance of [^{3}H]cholesteryl linoleate from NP-C plasma did not result in the anticipated faster appearance of free [^{3}H]cholesterol in plasma. In fact, the peak ^{3}H activity in plasma free cholesterol was much lower and occurred later in NP-C (~3000) min than in normal (<2000 min) subjects. Furthermore, the ^{3}H activity in plasma free cholesterol of NP-C subjects was generally half of the normal subjects from 400 to 2000 min; after 2000 min the plasma ^{3}H activity in NP-C subjects started to converge with normal subjects. This pattern implies that [^{3}H]cholesterol was temporarily sequestered at an intracellular site in NP-C subjects.

TABLE 1. Plasma cholesterol levels and plasma [^{3}H]cholesteryl linoleate kinetics

		Plasma Cholesterol		Plasma Cholesteryl Linoleate	Exponentials			
	n	Total	Free		λ1	Y-Intercept 1	λ2	Y-Intercept 2
		mg/dl		%				
NP-C #1	20	148	37	58	0.00096	0.78	0.00008	0.13
NP-C #2	24	117	34	63	0.00103	0.81	0.00011	0.08
NP-C #3	23	155	40	59	0.00097	0.97	0.00008	0.06
Mean		140	37	60	0.00099	0.85	0.00009	0.09
Normal #1	24	258	55	56	0.00075	0.61	0.00015	0.29
Normal #2	21	207	58	62	0.00062	0.85	0.00009	0.14
Normal #3	21	155	43	63	0.00083	0.67	0.00015	0.23
Mean		207	52	60	0.00073	0.71	0.00013	0.22

Exponentials were determined with the SAAM computer program; λ units are min^{-1}; Y-Intercept units are fraction of ^{3}H-does in plasma space; n, number of samples for concentration, percentage, and determination of exponentials.

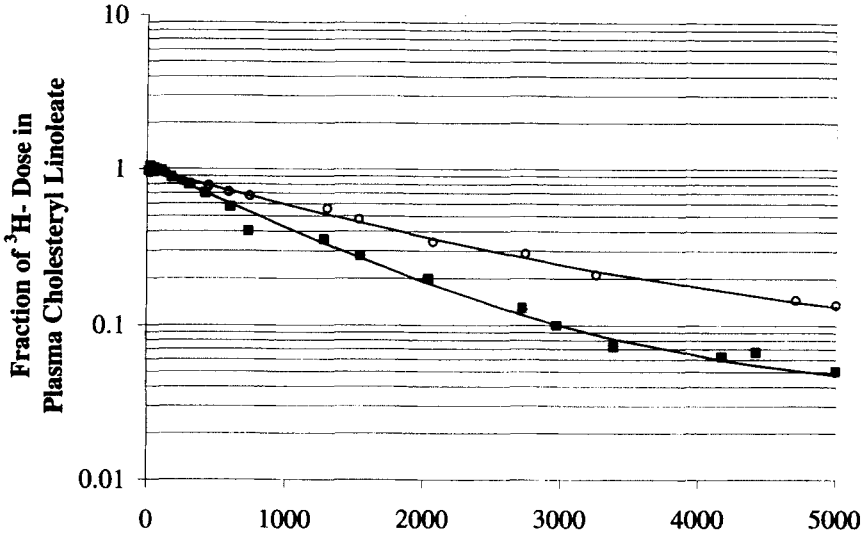

Fig. 1. ^{3}H-activity in plasma cholesteryl linoleate versus time after intravenous administration of [^{3}H]cholesteryl linoleate in autologous LDL. Representative subjects are shown; ○, Normal #2; ■, NP-C #3. Each line is from a 2 exponential fit. Total ^{3}H-activity administered and plasma volume estimated as 4.5% of the body weight in kg were used to calculate fraction of ^{3}H-dose in plasma.

Clues regarding the distribution of intracellular cholesterol to the plasma membrane were detected indirectly by monitoring free [^{3}H]cholesterol appearance in plasma during the initial 300 min after [^{3}H]cholesteryl linoleate administration in **Fig. 3**. From 0 to 25 min there was essentially no ^{3}H activity in plasma free cholesterol of any subject, excluding extracellular hydrolysis of [^{3}H]cholesteryl linoleate. The clues are *1*) from 25 to 90 min the ^{3}H activity in plasma free cholesterol increased rapidly in Normal #1, Normal #3, and NP-C #1, the three subjects administered [^{3}H]cholesteryl linoleate predominately in HDL; *2*) from 25 to 90 min the ^{3}H activity increased at a barely perceptible pace in Normal #2, NP-C #2, and NP-C #3, the three subjects administered [^{3}H]cholesteryl linoleate in LDL; and *3*) after 150 min (Figs. 2 and 3), ^{3}H activity in the three normal subjects increased more rapidly and after 250 min far exceeded that of all three NP-C subjects.

The two patterns of free [^{3}H]cholesterol appearance observed during the interval from 25 to 90 min, *1)* and *2*) above), appeared to be a function of whether [^{3}H]cholesteryl linoleate was administered in LDL versus HDL and not of NP-C versus normal. This interpretation assumes that equilibration of [^{3}H]cholesteryl linoleate among lipoproteins was not approached during the initial 90 min of the experiments. Equilibration is defined herein as when % distribution of [^{3}H]cholesteryl linoleate equals the % distribution of cholesteryl linoleate mass among lipoproteins. The distribution of cholesteryl linoleate ^{3}H activity and mass during the initial 300 min is shown in Table 2. There was gradual redistribution of [^{3}H]cholesteryl linoleate among all four lipoprotein classes but ^{3}H did not approach equilibration until after 300 min, strongly supporting this crucial assumption.

The data thus suggest that free [^{3}H]cholesterol reaches the plasma membrane sooner, as inferred by blood monitoring (Fig. 3), if derived from HDL rather than LDL [^{3}H]cholesteryl linoleate. The HDL pathway is not markedly altered in NP-C. In contrast, after about 90 min normally required for LDL-derived free cholesterol to reach the plasma membrane (clue *2* above) there is a marked decrease in LDL-derived free [^{3}H]cholesterol in NP-C plasma. It is even possible in NP-C #2 and #3 that nearly all free [^{3}H]cholesterol in plasma through 300 min is derived from HDL [^{3}H]cholesteryl linoleate after intravascular transfer from LDL. Comparison of Normals #1 and #3 with NP-C #1 in Fig. 3 are provocative in this regard; the divergence commencing after 90 min could reflect only HDL-derived free [^{3}H]cholesterol in NP-C #1 but both HDL- and LDL-derived free [^{3}H]cholesterol in Normals #1 and #3.

Free cholesterol is a major component of human bile which is secreted via the hepatocyte canalicular (apical) membrane. Bile was therefore monitored to gain information on intracellular traffic independent of the plasma data. **Figure 4** shows the appearance of free [^{3}H]cholesterol in bile during the entire time it was monitored after [^{3}H]cholesteryl linoleate administration. Comparison of each subject's bile data shown in Fig. 4 with plasma in Figs. 2 and 3 reveals that the ^{3}H specific activity of free cholesterol in bile exceeded that

TABLE 2. Distribution of cholesteryl linoleate ^{3}H-activity and mass among lipoproteins at various times after [^{3}H]cholesteryl linoleate administration

	% Distribution of Cholesteryl Linoleate			
	d ≤ 1.006 g/ml	1.006–1.019 g/ml	1.019–1.063 g/ml	1.063–1.21 g/ml
Normal #1				
^{3}H at time:				
0 (label)	0.0	0.1	21.6	78.0
6 min	1.7	0.5	30.3	67.5
67 min	5.2	1.1	28.4	65.3
187 min	4.7	2.3	36.9	56.1
290 min	4.8	2.8	39.0	53.5
Mass	2.6	1.8	69.2	26.4
Normal #2				
^{3}H at time:				
0 (label)	2.1	ND	96.4*	1.5
21 min	1.5	4.5	90.9	3.1
52 min	2.7	4.4	88.7	4.2
191 min	5.7	3.1	81.9	9.2
281 min	7.7	3.4	77.0	11.8
Mass	9.9	2.7	67.4	20.0
Normal #3				
^{3}H at time:				
0 (label)	0.1	0.1	7.4	92.4
10 min	2.0	0.7	10.4	86.9
58 min	5.1	1.3	15.1	78.5
150 min	8.6	2.1	22.4	66.9
280 min	9.5	3.1	33.2	54.2
Mass	5.7	1.8	62.0	30.5
NP-C #1				
^{3}H at time:				
0 (label)	0.0	0.2	14.1	85.6
15 min	4.5	0.7	12.2	82.6
72 min	12.5	1.8	17.2	68.5
182 min	13.1	3.8	29.3	53.8
297 min	13.5	4.1	39.6	42.7
Mass	8.4	2.4	59.6	29.6
NP-C #2				
^{3}H at time:				
0 (label)	2.0	ND	91.2*	6.8
25 min	1.8	6.7	84.2	7.3
51 min	2.7	6.2	80.9	10.2
168 min	4.1	5.9	75.8	14.2
293 min	5.6	6.0	70.9	17.5
Mass	8.2	7.6	57.3	26.9
NP-C #3				
^{3}H at time:				
0 (label)	1.1	ND	95.9[a]	2.8
24 min	2.1	2.8	91.8	3.3
51 min	2.8	2.7	89.9	4.6
177 min	5.4	2.1	83.4	9.1
298 min	7.6	2.2	79.1	11.1
Mass	11.5	3.1	64.4	21.0

Lipoproteins were isolated by ultracentrifugation at the densities shown. Cholesteryl linoleate was isolated by HPLC. Each mass value is the average of 5 plasma samples; the SEM for each subject's mass was ≤3%. The results for ^{3}H at time 0 are from an aliquot of the ^{3}H-labeled preparation. Lipoproteins from a plasma sample obtained after about 1000 min revealed the distribution of ^{3}H-activity in cholesteryl linoleate was the same as that of mass (equilibration) of each subject; ND, not done.

[a] 1.006–1.063 g/ml.

in plasma at all times during the 400–500 min of bile collection. This observation strongly suggests that most of the free [^{3}H]cholesterol in bile originated from cholesteryl ester hydrolysis in the liver cell.

As was seen in plasma (Fig. 3), the ^{3}H activity in bile free cholesterol (Fig. 4) increased rapidly between 25 and 90 min in all three subjects (Normals #1 and #3, and NP-C #1) administered [^{3}H]cholesteryl linoleate in HDL. After 90 min in Fig. 4, the bile ^{3}H activity increased further in Normal #1 and #3, but tapered off in NP-C #1. This divergence probably occurred because of intravascular transfer of [^{3}H]cholesteryl linoleate to LDL in all three subjects, but only in normals did the LDL-derived free [^{3}H]cholesterol have ready access to the plasma membrane. As in plasma, the ^{3}H activity in bile (Fig. 4) increased very little from 0 to about 100 min in the three subjects (Normal #2, NP-C #2 and #3) administered LDL. After about 120 min in Fig. 4, secretion of bile [^{3}H]cholesterol activity accelerated in Normal #2, probably derived from [^{3}H]cholesteryl linoleate that was both injected in LDL and transferred to HDL. Relatively little ^{3}H was secreted in bile of NP-C #2 and #3 after 120 min in Fig. 4, and the ^{3}H was probably derived only after transfer of injected LDL-label to HDL.

Bile cholesterol is reflective of cholesterol in canalicular membranes whereas plasma presumably mirrors the basolateral membrane. The similar ^{3}H response in plasma (Fig. 3) and bile (Fig. 4) therefore indicates rapid mixing of free cholesterol among both plasma membrane domains and/or the same mechanism of intracellular distribution to both domains.

Monitoring bile confirmed that free cholesterol normally arrives at the plasma membrane faster if derived from HDL than LDL cholesteryl linoleate implying a delay of about 90 min required for cellular processing of LDL. The slow rate (after the delay) of ^{3}H appearance in NP-C plasma and bile free cholesterol derived from endocytosed LDL [^{3}H]cholesteryl linoleate suggested a lysosomal specific trafficking defect. However, it did not permit exclusion of a generalized lesion in cellular cholesterol trafficking. This question was resolved by monitoring ^{14}C appearance in plasma and bile free cholesterol newly synthesized from [^{14}C]mevalonate jointly with ^{3}H appearance in each subject. As shown in **Fig. 5**, no difference was noted between normal and NP-C subjects, even during the initial 300 min. Similarly, **Fig. 6** shows no difference in the appearance of free [^{14}C]cholesterol in bile between normal and NP-C subjects. Thus, no evidence was found for a general trafficking lesion.

In each subject the ^{14}C specific activity of free cholesterol in bile (Fig. 6) exceeded that of plasma (Fig. 5, insert) from 0 to 300 min, showing that most of the free [^{14}C]cholesterol in bile was produced in the hepatocyte

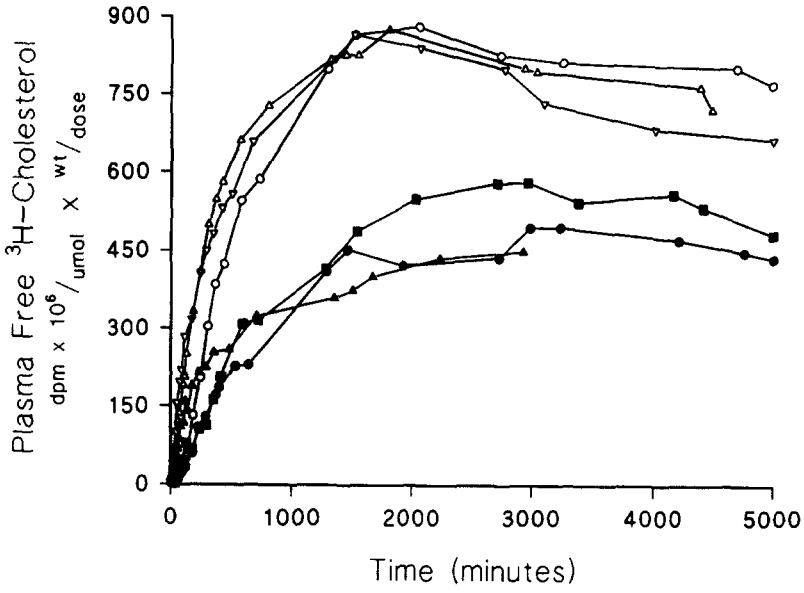

Fig. 2. ^{3}H-activity in plasma free cholesterol versus time after intravenous administration of ^{3}H-cholesteryl linoleate. [^{3}H]cholesteryl linoleate was given in LDL or predominately in HDL, shown in parentheses for each subject as follows: Normal #1 (HDL) = △; Normal #2 (LDL) = ○; Normal #3 (HDL) = ▽; NP-C #1 (HDL) = ▲; NP-C #2 (LDL) = ●; NP-C #3 (LDL) = ■.

and not derived by transfer via plasma of free [^{14}C]cholesterol produced elsewhere.

Bile lipids were analyzed as the composition of bile in humans with NP-C has not been reported. No abnormalities were noted. In all six subjects, phosphatidylcholine comprised >90% of total phospholipid and 1-palmitoyl 2-linoleoyl was the major (49–54%) molecular species (14, 19). Total bile acid concentration in the bile of Normals #1, #2, and #3 and NP-C #1, #2, and #3 was 9.1, 17.7, 45.0, 19.0, 26.9, and 31.2 μmol/ml, respectively; the molar ratio of total bile acid/cholesterol was 16.9, 20.1, 28.7, 30.4, 29.0, and 27.2, respectively. Bile acid composition was similar among the six subjects; cholic acid comprised 44–58%, chenodeoxycholic acid 23–33%, deoxycholic acid 17–27% and other bile acids <2%.

Interpretation of ^{3}H and ^{14}C appearance in bile acids versus time was problematic because of recycling via the enterohepatic circulation which can be pulsatile. We found that comparison of bile acid data expressed as dpm/umol was also difficult (data not shown) probably due to variation in bile acid synthetic rate and pool size from subject to subject. Because traffic of newly synthesized [^{14}C]cholesterol was similar among NP-C and normal subjects (Figs. 5 and 6), the ratio of ^{3}H/^{14}C in bile acids versus time was examined, **Figure 7**, assuming the denominator (^{14}C) was constant from subject to subject. The ^{3}H/^{14}C ratio in bile cholesterol is also

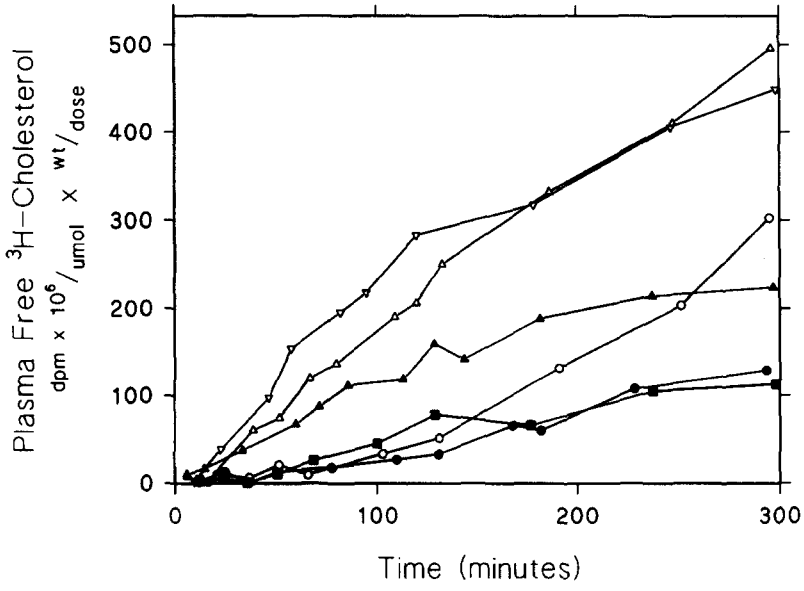

Fig. 3. ^{3}H-activity in plasma free cholesterol versus time during the initial 300 min after [^{3}H]cholesteryl linoleate administration. The data is from the 0 to 300 min interval in Fig. 2 but with the time scale expanded. Symbols as in Fig. 2 legend.

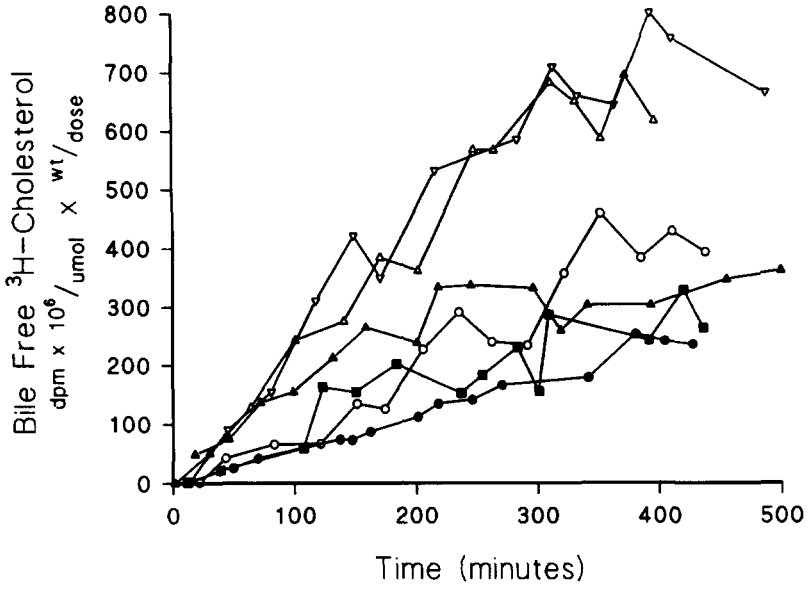

Fig. 4. ^{3}H-activity in bile free cholesterol versus time after intravenous administration of [^{3}H]cholesteryl linoleate in LDL or predominantly in HDL. Symbols as in Fig. 2. legend.

shown in Fig. 7 for comparison; the ^{3}H/^{14}C pattern in bile cholesterol is similar to that seen in Fig. 4, lending some validity to the use of ratios.

As shown in Fig. 7, the ^{3}H/^{14}C ratio versus time in cholic acid followed the same pattern as that in chenodeoxycholic acid. We expected the ^{3}H/^{14}C ratio in both bile acids to be somewhat lower than that in bile cholesterol because about 5% of primary bile acids, but no bile cholesterol, are derived directly from newly synthesized [^{14}C]cholesterol (6). This expectation was met in Normal #1 and NP-C #1, the subjects administered [^{3}H]cholesteryl linoleate in HDL. However, in the three subjects (Normal #2, NP-C #2 and NP-C #3) administered ^{3}H in LDL, the bile acid ^{3}H/^{14}C ratios exceeded the ratios in bile cholesterol and there were no differences among the ratios of these three subjects. These observations suggest that lysosomal free [^{3}H] cholesterol derived from LDL may bypass the plasma membrane and be used directly for bile acid synthesis.

As an in vitro correlate to these in vivo studies, LDL cholesterol metabolism was compared in cultured fibroblasts from the same three NP-C subjects and two separate normal controls. After a period of cholesterol deprivation, cultures were incubated with LDL and [^{3}H]oleate. [^{3}H]triglyceride synthesis was comparable in all cell lines at 3, 6, and 24 h (data not shown). However, cholesteryl[^{3}H]oleate formation, **Table 3**, was markedly reduced in NP-C #1 and #2 and moderately

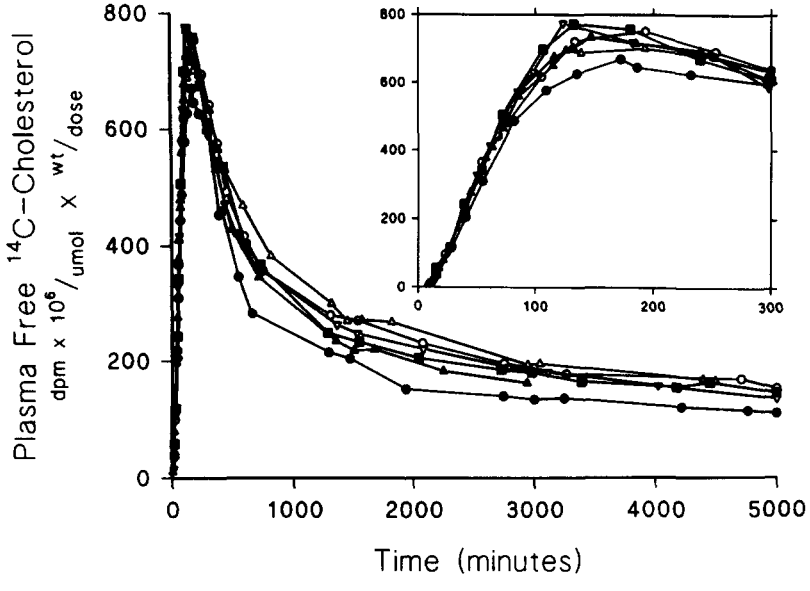

Fig. 5. ^{14}C-activity in plasma free cholesterol versus time after intravenous administration of [^{14}C]mevalonic acid. Insert shows the same data with the time scale expanded form 0 to 300 min. [^{14}C]mevalonic acid was administered simultaneously with [^{3}H]cholesteryl linoleate. Symbols as in Fig. 2 legend.

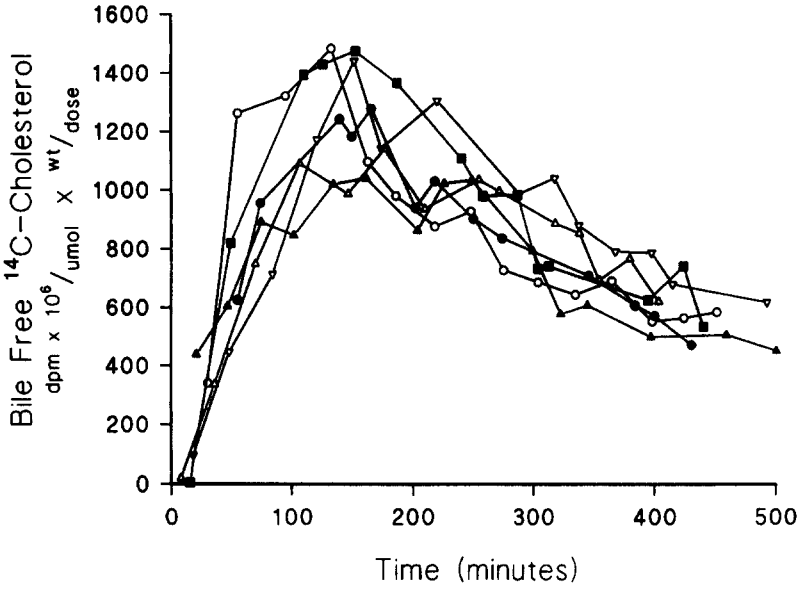

Fig. 6. ^{14}C-activity in bile free cholesterol versus time after intravenous administration of [^{14}C]mevalonic acid. Symbols as in Fig. 2 legend.

reduced in NP-C #3. The intracellular distribution of LDL-derived cholesterol was monitored cytochemically in the same cultures. After addition of LDL to the medium for 24 h, the fibroblast cell lines were stained with filipin, a fluorescent probe that complexes with free cholesterol. In the normal cells (**Fig. 8A**) there is plasma membrane and light scattered intracellular staining. NP-C #1 and #2 show intense staining of perinuclear structures previously identified as lysosomes and poor staining of the plasma membrane. In NP-C #3, Fig. 8D, the perinuclear structures show staining intermediate between normal and NP-C #1 and #2. The in vivo results for NP-C #3 in Fig. 2 also show an intermediate defect in this subject suggesting that the in vitro and in vivo assays are measuring related phenomenon.

DISCUSSION

Niemann-Pick C disease (NP-C) is an inborn error characterized cytochemically by cellular lipidosis and biochemically by accumulation of free cholesterol in lysosomes (1). Sphingomyelinase and cholesteryl ester hydrolase activities are near normal, distinguishing NP-C as a unique disorder. The NP-C cellular lesion is highlighted in vitro by a culture system that features cholesterol deprivation followed by a period up to 24 h of maximized LDL uptake (20). Under these artificial conditions, the consequences of delayed lysosomal cholesterol mobilization are maximized and feature long delays in distribution of free cholesterol to the plasma membrane and induction of homeostatic responses (1, 20, 21). This experimental approach utilizes an extreme and sequential perturbation of cholesterol availability to cells and has limited in vivo relevance.

To explore the physiologic significance of the NP-C lesion, it has become important to establish a relevant in vivo setting for the phenotypic expression of the mutation. We reasoned that in a steady-state free of fluctuations in lipoprotein availability, cholesterol-mediated homeostatic responses are likely to be normal in NP-C subjects. One could even expect that the amount of free cholesterol leaving the lysosomes of NP-C cells in vivo would nearly equal that of normal cells but at the expense of an abnormally high lysosomal content, reflecting the genetically determined low fractional turnover rate of cholesterol exiting these organelles. Under such circumstances the circulating lipoprotein profile might be unremarkable, as has been reported in NP-C subjects (1). Consequently, we endeavored to identify the NP-C mutation in vivo by first administering [^{3}H]cholesteryl linoleate in autologous LDL or HDL by vein. Then we monitored the rate of free [^{3}H]cholesterol movement from inside the cell, derived from endocytosed [^{3}H]cholesteryl linoleate, to the plasma membrane by taking frequent samples of plasma and bile. Free cholesterol is known to exchange rapidly between basolateral membranes and plasma lipoproteins (5, 6), and bile cholesterol is largely derived from canalicular membranes (22). Plasma and bile should, therefore, serve as proxies in vivo for these membrane domains. Appearance of isotopic cholesterol in the media has been shown in cell culture to mirror that in the plasma membrane (23, 24).

Compared to normal subjects, the rate of LDL-derived free [^{3}H]cholesterol movement to the plasma membrane of NP-C subjects, as measured by its appear-

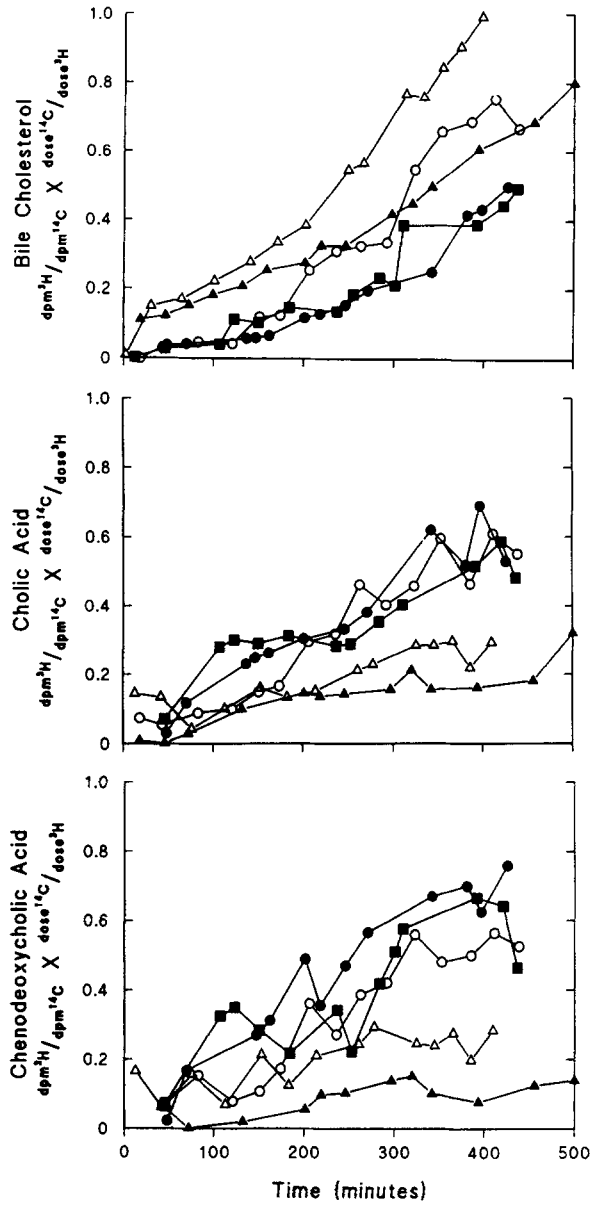

Fig. 7. The $^{3}H/^{14}C$ ratio (normalized for doses) versus time in bile cholesterol (top panel), cholic acid (middle), and chenodeoxycholic acid (bottom). At 0 min, [^{3}H]cholesteryl linoleate in autologous LDL (○, ●, ■) or predominantly in HDL (△, ▲) was administered by vein simultaneously with [^{14}C]mevalonic acid. Symbols as in Fig. 2 legend. Bile acid radioactivity not determined in Normal #3.

ance in plasma and bile, was markedly diminished. The peak specific activity (dpm/umol) of free [^{3}H]cholesterol in plasma occurred 1000 min later and was 50% lower in NP-C (Fig. 2). This was not due to dilution of free [^{3}H]cholesterol in an expanded pool of plasma free cholesterol in NP-C as plasma free cholesterol concentration was in fact lower in NP-C than in normal subjects. Nor was there evidence for marked expansion of the rapidly miscible pool of cholesterol (25) in NP-C as the disappearance of [^{14}C]cholesterol from blood was similar in all subjects. The diminished rate of free [^{3}H]cholesterol appearance in the plasma and bile of NP-C subjects was not secondary to slow clearance of [^{3}H]cholesteryl linoleate from blood as clearance proved to be 35% faster in NP-C. Defective lysosomal hydrolysis of LDL cholesteryl ester is not likely to represent a contributory factor as filipin staining of each NP-C subjects' fibroblasts showed free cholesterol accumulation in lysosomes. The diminished rate of free [^{3}H]cholesterol movement to the plasma membrane of NP-C subjects was not due to a generalized defect in intracellular sterol traffic. The appearance of newly synthesized free [^{14}C]cholesterol, labeled via [^{14}C]mevalonic acid, in the plasma membrane as mirrored by plasma and bile was the same in NP-C as in normal subjects. This finding is consistent with the report of Liscum, Ruggiero, and Faust (26) using cultured NP-C fibroblasts and [^{3}H]acetate to label synthesized cholesterol. Thus the data are most compatible with a unique defect in transport of free cholesterol from lysosome or closely related organelle to the plasma membrane in NP-C subjects.

A schematic presentation of intracellular cholesterol distribution deduced from the present studies and encompassing current concepts is presented in **Fig. 9**. The NP-C mutation is depicted as blocking relocation of endocytosed LDL-derived free cholesterol to the plasma membrane. This conclusion is consistent with the present data and supported by in vitro studies characterizing the lesion (1, 3, 20, 21, 26). Our data in NP-C subjects furthermore indicate that the lysosomal pool of free cholesterol is dynamic and not stagnant as lysosomal-generated cholesterol does reach the plasma membrane, albeit more slowly, for exchange into plasma. The lack of a complete block in transfer may suggest that an alternate route not affected by the mutation is able to contribute to the removal of cholesterol from lysosomes. A potential for therapeutic intervention may lie in further delineating the alternate route with the hope for up-regulating such a putative mechanism. Compartmentalized kinetic modeling of the present data will allow measurement of the size and turnover rate of the NP-C-induced lysosomal cholesterol pool and may allow us to readily evaluate prospective therapeutic interventions through quantitation of lipoprotein cholesterol processing as depicted in the present study.

Three additional findings featured in Fig. 9 came to light from the present studies. First, in the normal and NP-C subjects administered [^{3}H]cholesteryl linoleate in LDL, essentially no free [^{3}H]cholesterol appeared in extracellular pools during the initial 90 min, suggesting that

TABLE 3. LDL-stimulated cholesteryl ester synthesis in cultured fibroblasts

Cell Line	Cholesteryl [^{3}H]Oleate Formation		
	3 h	6 h	24 h
		pmol/mg protein	
Normal #A	112 ± 16	3,975 ± 146	76,021 ± 3289
Normal #B	219 ± 12	3,622 ± 194	60,669 ± 2835
Mean	166	3,798	68,345
NP-C #1	0 ± 10 (<1%)	0 ± 8 (<1%)	3,294 ± 160 (5%)
NP-C #2	0 ± 7 (<1%)	2 ± 7 (<1%)	1,435 ± 88 (2%)
NP-C #3	26 ± 14 (16%)	1,162 ± 63 (31%)	46,233 ± 1208 (68%)

Stock fibroblasts were incubated for 3 days in EMEM medium containing 10% LPDS. The cholesterol-depleted cells were harvested by trypsinization and plated at 100,000 cells/35-mm well. Cells were cultured for a further 24 h in LPDS medium. Cultures were subsequently incubated for indicated times with fresh medium containing LDL (50 μg/ml) and 100 μM[^{3}H]oleate (200 dpm/pmol). Cell monolayers were washed with cold PBS and lipids were extracted with 0.5 ml isopropanol. Incorporation of [^{3}H]oleate into newly synthesized cholesteryl esters was measured by thin-layer chromatography; (%) is that of normal mean.

in all subjects receptor-mediated endocytosis and lysosomal processing require a finite time approaching 90 min. This processing time agrees generally with results of in vitro studies (10, 27).

Second, three subjects (NP-C #1, Normal #1, and Normal #3) were administered [^{3}H]cholesteryl linoleate in HDL. Although exchange among lipoproteins occurred during its residency in the blood, there was a window of at least 100 min after administration during which a large majority of the [^{3}H]cholesteryl linoleate remained in HDL. In these three subjects, including the one with NP-C, free [^{3}H]cholesterol appeared rap-

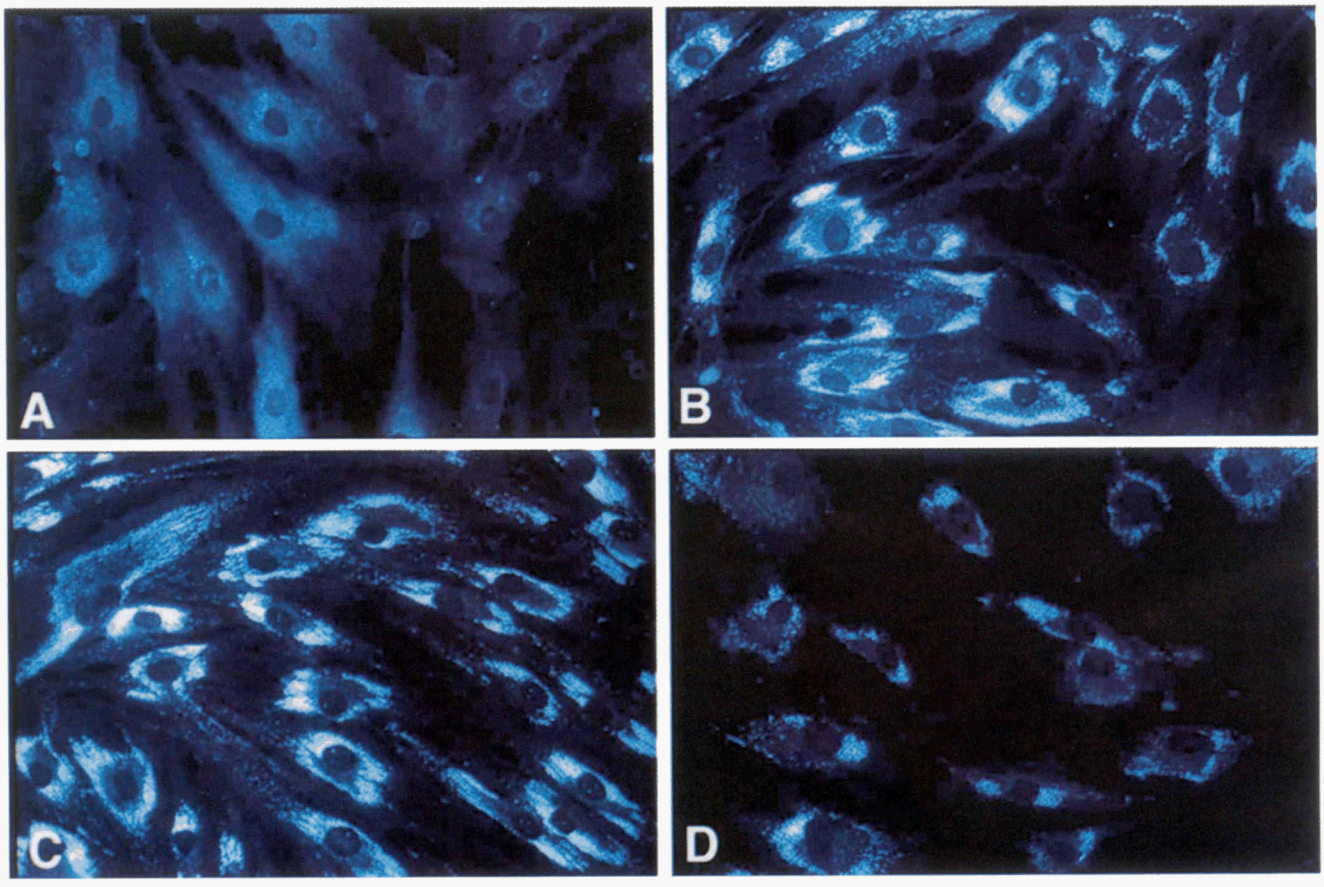

Fig. 8. Filipin-stained fibroblasts showing localization of free cholesterol after incubation with LDL for 24 h as described in Table 3. Panel A: normal #A. Panel B: NP-C #1. Panel C: NP-C #2. Panel D: NP-C #3.

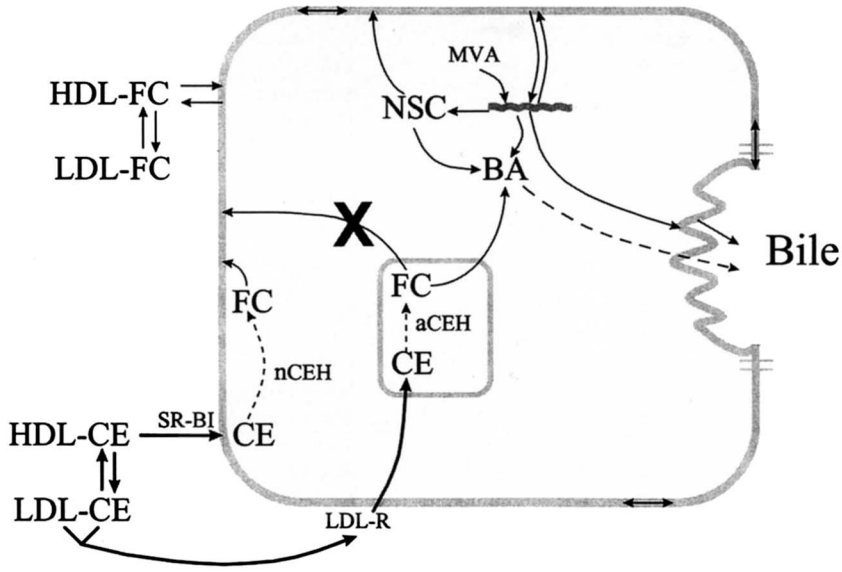

Fig. 9. Proposed schema of cholesterol distribution in a human hepatocyte derived from the present study and selected current concepts. **X**, site of defect in NP-C. nCEH and aCEH, neutral and acidic cholesteryl ester hydrolase, the latter depicted in a lysosome; →, free cholesterol transport; ➞, cholesteryl ester transport; ⇢, cholesteryl ester hydrolysis; ↔, free cholesterol lateral movement and flip-flop in the plasma membrane, both of which are presumed to be very rapid. BA, bile acid; the arrows leading to BA represent bile acid synthesizing enzymes. MVA, mevalonic acid. NSC, newly synthesized cholesterol. LDL-R, LDL receptor. SR-BI, scavenger receptor, class B, type I, representing a possible mechanism for cholesteryl ester uptake from HDL (36). Endoplasmic reticulum and canalicular (apical) membrane domains also shown.

idly and at equivalent rates in plasma (and in bile) during the initial 25 to 90 min after administration (Fig. 3 and 4). This provides the first in vivo evidence in humans for cellular uptake of cholesteryl esters from HDL. Pittman and coworkers (28, 29) previously showed selective uptake of esters from HDL in cell culture and animal tissues. Although our data obtained in vivo do not distinguish selective ester uptake from HDL particle uptake, our data do show that the pathway involves extralysosomal hydrolysis and rapid appearance of the released free cholesterol product in the plasma membrane, consistent with in vitro findings (30). Presumably because this pathway avoids cycling of cholesterol through lysosomes, it remains impervious to the NP-C mutation.

Third, in the normal and NP-C subjects administered [^{3}H]cholesteryl linoleate in LDL there was a disproportionate amount of [^{3}H]cholesterol converted to bile acids relative to newly synthesized [^{14}C]cholesterol converted. This finding might imply direct routing of a small fraction of lysosomal free cholesterol to microsomal or mitochondrial bile acid synthesizing enzymes that bypass the mutation, possibly by direct contact of respective organelles. Precedent for translocation of a portion of lysosomal cholesterol to the endoplasmic reticulum by a plasma membrane-independent route has been established in vitro for esterification (31, 32). Alternatively, free cholesterol in lysosomes could be oxidized directly to oxysterols. Several oxysterols are prime substrates for bile acid synthesis (33).

Because analysis of bile was a feature of our experimental design and because free cholesterol in plasma exchanges most rapidly with hepatocytes (6), our conclusions apply largely to hepatocytes. The liver is quantitavely the major site of LDL endocytosis (34) and might be expected to take the brunt of the load in NP-C. However, after the neonatal period, liver disease is rarely a major feature in NP-C subjects (1). Perhaps when the NP-C infant's bile acid synthetic pathway fully matures the hepatic lysosomal load of free cholesterol can be reduced enough to avoid liver damage by direct routing to bile acid synthesizing enzymes. The major problem after infancy is neurologic. LDL is absent from the nervous system but apoE-rich lipoproteins are present as are receptors for apoE which would probably process lipoprotein ligands through the lysosomal pathway (35).

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