Lysosomal unesterified cholesterol content correlates with liver cell death in murine Niemann-Pick type C disease

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Abstract **Niemann-Pick type C (NPC) disease is a multisystem disorder resulting from mutations in the *NPC1* gene that encodes a protein involved in intracellular cholesterol trafficking. Significant liver dysfunction is frequently seen in patients with this disease. The current studies used *npc1* mutant mice to investigate the association between liver dysfunction and unesterified cholesterol accumulation, a hallmark of NPC disease. Data from 92 *npc1*$^{-/-}$ mice (age range, 9–56 days) revealed a significant positive correlation between the plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and whole liver cholesterol content. In 56 day old *npc1*$^{-/-}$ mice that had been fed from 35 days of age a rodent diet or the same diet containing either cholesterol (1.0%, w/w) or ezetimibe (a sterol absorption inhibitor; 0.0125%, w/w), whole liver cholesterol content averaged 33.5 ± 1.1, 87.9 ± 1.7, and 20.8 ± 0.9 mg, respectively. Again, plasma ALT and AST activities were positively correlated with hepatic cholesterol content. In contrast, plasma transaminase levels remained in the normal range in *npc1*$^{+/+}$ mice, in which hepatic esterified cholesterol content had been increased by 72-fold by feeding a high-cholesterol, high-fat diet. These studies suggest that the late endosomal/lysosomal content of unesterified cholesterol correlates with cell damage in NPC disease.**—Beltroy, E. P., B. Liu, J. M. Dietschy, and S. D. Turley. **Lysosomal unesterified cholesterol content correlates with liver cell death in murine Niemann-Pick type C disease. *J. Lipid Res.* 2007.** 48: **869–881.**

Supplementary key words hepatic dysfunction • chylomicron cholesterol • low density lipoprotein receptor • biliary bile acid • hepatomegaly • small intestine

At least two proteins, designated Niemann-Pick type C1 (NPC1) and NPC2, are involved in the movement of cholesterol and, possibly, other lipids from the late endosomal/lysosomal compartment to the metabolically active pool of sterol in the cytosolic compartment of all cells (1–3). Mutations in either of these proteins cause marked abnormalities in cholesterol trafficking in every tissue (4), and in humans, this dysfunction gives rise to the clinical syndrome known as Niemann-Pick type C (NPC) disease (5–7). These individuals may present with evidence of serious involvement of at least three different organ systems: the liver, lungs, and central nervous system.

The earliest clinical findings are usually associated with liver abnormalities. More than half of the affected infants apparently have cholestasis with prolonged neonatal jaundice and hepatosplenomegaly. Hepatic histology is often interpreted as "resolving hepatitis" or "giant cell hepatitis." Although the jaundice resolves in many of these infants, a subset goes on to develop chronic liver function abnormalities, cirrhosis, and even liver failure (8–10). Less commonly, the complications of portal hypertension and hepatocellular carcinoma occur (11). These patients may also present with recurrent pulmonary symptoms and even die of acute pulmonary failure. Histologically, these clinical symptoms are associated with extensive pulmonary infiltration by lipid-laden macrophages that is referred to as "endogenous lipid pneumonia" (12–15). If the affected children escape these hepatic and pulmonary complications, they invariably develop evidence of progressive neurodegeneration. Symptoms may begin in late infancy or early childhood, with mild intellectual impairment and poor performance in school, and progress to dysarthria, ataxia, abnormal vertical gaze, and dementia (5, 7, 16, 17).

More than 20 years ago, it was shown that this NPC disease could be differentiated from other lysosomal storage diseases by the finding that fibroblasts from these children did not readily synthesize cholesteryl esters after exposure to LDLs (18). Since then, the nature of the defect in cholesterol trafficking caused by the loss of function of NPC1 protein has been described in detail and is summarized in **Fig. 1**. Most cells express low density

Abbreviations: ALT, alanine aminotransferase; apoE, apolipoprotein E; AST, aspartate aminotransferase; bw, body weight; CMr, chylomicron remnant; LDLR, low density lipoprotein receptor; NPC, Niemann-Pick type C; NPC1L1, Niemann-Pick C1-Like 1; VLDLr, very low density lipoprotein remnant.

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Manuscript received 14 November 2006 and in revised form 11 January 2007.

Published, JLR Papers in Press, January 14, 2007.
DOI 10.1194/jlr.M600488-JLR200

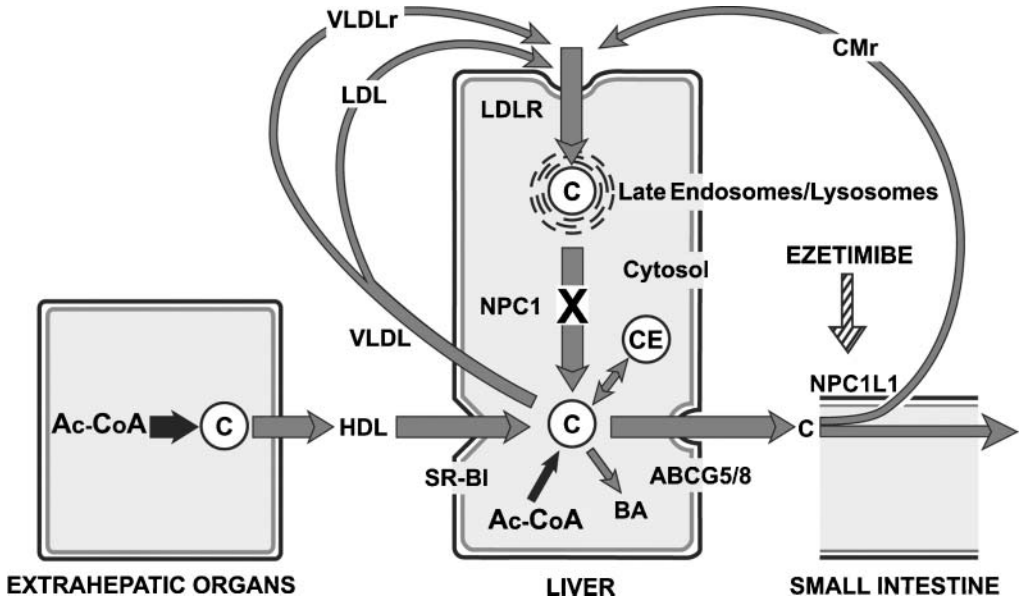

Fig. 1. Diagram of the net flow of cholesterol through the various tissues of the mouse and the site of action of ezetimibe, a sterol absorption inhibitor. Cholesterol, carried in LDL and the remnants of very low density lipoproteins (VLDLrs) and chylomicrons (CMrs), is taken up into the liver by the low density lipoprotein receptor (LDLR) and processed through the clathrin-coated pit. Mutations in Niemann-Pick type C1 (NPC1) cause entrapment of unesterified cholesterol in the late endosomal/lysosomal compartment. This diagram shows two intracellular pools of unesterified cholesterol (C), one within the late endosomal/lysosomal compartment and a second that is metabolically active within the cytosol. This latter pool can be used for membrane sterol turnover and excretion into the bile and for the synthesis of cholesteryl esters (CE) and bile acids (BA). The sterol absorption inhibitor, ezetimibe, acts to block the sterol transporter, Niemann-Pick C1-Like 1 protein (NPC1L1), and so interrupts the enterohepatic circulation of cholesterol (66). Ac-CoA, acetyl-coenzyme A; SR-BI, scavenger receptor class B type I. This diagram was taken from ref. 31 and modified.

lipoprotein receptors (LDLRs) that can bind lipoproteins carrying apolipoprotein E (apoE) with very high affinity as well as those carrying apoB-100 with lesser affinity (19–22). The density of these receptors is highest in the endocrine tissues and liver, but they can also be detected in most other organs (23, 24). However, the tight endothelial lining of most capillary beds limits the access of circulating lipoproteins to these LDLRs in most organs, including the central nervous system (25). The concentration of LDL in peripheral lymph, for example, is only 5–10% of that found in the plasma (26, 27).

Unlike most of these tissues, however, the sinusoidal capillaries of the liver are fenestrated, with openings of 50–90 nm in diameter, so that even the large remnants of very low density lipoproteins (VLDLrs) and chylomicrons (CMrs), as well as LDLs, have direct access to the LDLRs on hepatocytes (28). As a consequence, essentially all of these apoE-containing VLDLrs and CMrs, as well as ~70% of the LDLs, are cleared from the plasma into the liver in all species that have been studied (23, 29). Once the bound lipoproteins are endocytosed, the various lipids, including cholesteryl esters, are hydrolyzed in the acidic environment of the late endosomes/lysosomes. The unesterified cholesterol is then transferred, using the activities of both NPC2 and NPC1, to the metabolically active pool of sterol in the cytosol. As also illustrated in Fig. 1, cholesterol that reaches the liver through the HDL/scavenger receptor class B type I pathway or that is synthesized locally in the liver reaches this metabolically active pool of sterol without passing through the late endosomal/lysosomal compartment (30).

From these considerations, it is not surprising that the liver bears the brunt of a mutation that inactivates NPC1. Under these conditions, the concentration of unesterified cholesterol in this organ increases throughout life, the weight of the liver increases, and there is biochemical evidence of hepatocyte damage, as manifest by increases in the plasma alkaline phosphatase and transaminase levels (4, 31, 32). However, despite the block in the movement of unesterified cholesterol out of the late endosomal/lysosomal compartment in cells of the liver and other tissues, there is no shortage of sterol in the metabolically active pool of these cells. This is because de novo synthesis increases to supply adequate amounts of cholesterol for the turnover of membrane sterol and for the synthesis of bile acids and, in the endocrine glands, steroid hormones (4, 24, 30). In the mouse with this mutation, for example, total body cholesterol synthesis increases from ~120 mg/day/kg body weight (bw) in the normal animal to 182 mg/day/kg. This increase in synthesis just offsets the 67 mg/day/kg unesterified cholesterol that is sequestered in the late endosomal/lysosomal compartment of cells in

the liver and other organs of the body and is thus unavailable for metabolic use (30).

Even though this adaptive increase in synthesis prevents a shortage of intracellular sterol, the accumulation of unesterified cholesterol in the late endosomal/lysosomal compartment is associated with cell death in the liver, central nervous system, and, probably, other organs. In both the liver and brain, for example, mRNA levels of various caspaces are increased and there is evidence of cell death through apoptosis (31, 33, 34). However, there is considerable uncertainty regarding which organelle in the cell gives rise to the apoptotic signal and, furthermore, which chemical species in that organelle initiates the process. Part of the difficulty of resolving this question is that unesterified cholesterol is a very hydrophobic amphipath that strongly interacts through hydrophobic bonding with other lipids, particularly lipids such as sphingomyelin and certain glycosphingolipids. Such molecules come together, for example, in highly ordered, raft-like microdomains in membranes that cannot be disrupted by detergents (35). These avid hydrophobic interactions may explain why in a disorder such as NPC disease there is also an accumulation of glycosphingolipids in cells in which the primary defect appears to be in the transport of cholesterol (36, 37). Conversely, in Niemann-Pick type A disease, there is also an accumulation of unesterified cholesterol under circumstances in which the primary defect is in the catabolism of sphingomyelin (38).

The murine model of NPC disease, the *npc1*$^{-/-}$ BALB/c mouse, provides a unique opportunity to explore this problem in detail. In this model, the amount of unesterified cholesterol reaching the late endosomal/lysosomal compartment of cells in the liver can be controlled and varied from very low amounts, by blocking the activity of the intestinal sterol transporter, Niemann-Pick C1-Like 1 (NPC1L1), to very high amounts, by enriching the CMr with exogenous cholesterol. The relationship between this expansion of the ectopic intracellular cholesterol pool and subtle cellular damage can then be measured using various liver function tests. Together, the results of these experiments strongly suggest that it is, indeed, the magnitude and duration of the expansion of the pool of unesterified cholesterol in the late endosomal/lysosomal compartment that somehow initiates liver cell damage. By inference, this finding further suggests that the signal for this damage likely comes from the late endosomal/lysosomal compartment of these cells.

MATERIALS AND METHODS

Animals and diets

Wild-type (*npc1*$^{+/+}$) and homozygous mutant (*npc1*$^{-/-}$) mice were generated from heterozygous *npc1*$^{+/-}$ animals with a BALB/c background (1, 4, 31). All animals except the heterozygous breeding stock were fed ad libitum a cereal-based, low-cholesterol rodent diet (No. 7001; Harlan Teklad, Madison, WI) after weaning at 19 days of age. This diet had a cholesterol content of 0.02% (w/w) and a minimum crude fat content of 4% (w/w). The breeding stock were maintained on another formulation (Harlan Teklad No. 7002) that had cholesterol and minimum crude fat contents of 0.03% (w/w) and 6.0% (w/w), respectively. In several studies, the meal form of diet 7001 was used to prepare experimental diets containing either cholesterol (0.25, 0.50, or 1.00%, w/w) or ezetimibe (Schering-Plough Research Institute, Kenilworth, NJ) (0.00125, 0.00625, or 0.0125%, w/w) (39). These dietary levels of ezetimibe provided doses of ~2, 10, and 20 mg/day/kg bw, respectively. In some experiments, ezetimibe was administered orally to pups every day before they were weaned, starting as early as the day of birth. In these cases, the ezetimibe was suspended in medium-chain triglycerides (20 mg/ml; Mead Johnson Nutritionals, Evansville, IN) and administered using an adjustable-volume Gilson Pipetman P20 pipet (Gilson, Inc., Middleton, WI). Suckling pups treated with ezetimibe before 19 days of age were weighed and administered the ezetimibe suspension (1 μl/g bw) every 24 h. The aliquot of suspension was pipeted directly onto the back of the tongue of the pup while it was held upright, and then a swallowing reflex was induced. The pups were then immediately returned to their parents. This procedure provided an approximate dose of 20 mg/day/kg bw.

All pups were routinely weaned and genotyped at 19 days of age unless stated otherwise. In those experiments in which pups were studied before 19 days of age, they were genotyped on the day of study. After weaning, oral dosing was discontinued and the treated mice received ezetimibe in their diet as described above until 56 days of age unless specified otherwise. In one study, *npc1*$^{-/-}$ mice that had received ezetimibe from birth remained on treatment for their entire lifespan, which averaged 82 days. Matching *npc1*$^{+/+}$ animals from this same study were kept on ezetimibe treatment until 112 days of age, at which time they were used for the measurement of plasma liver enzyme activities. Unless stated otherwise, every experimental group consisted of equal or nearly equal numbers of males and females. All mice were housed in groups on wood shavings in plastic colony cages in a light-cycled room and were studied in the fed state toward the end of the dark phase of the lighting cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Measurement of total cholesterol content in liver, extrahepatic tissues, and whole animals

The mice were exsanguinated via the inferior vena cava into heparinized syringes. The whole liver was removed, rinsed in 0.9% (w/v) NaCl solution, blotted, and weighed. Aliquots of liver were added to alcoholic KOH. In one experiment, spleen, kidney, lung, brain, and residual carcass were also taken. In those experiments in which whole body cholesterol content was determined, the entire gastrointestinal tract was removed, washed out, and added back to the carcass in alcoholic KOH. After saponification of the tissue aliquots and remaining animal, cholesterol was extracted and quantitated by gas chromatography using stigmastanol as an internal standard (40, 41). Except in one case, hepatic cholesterol content was always expressed as mg/whole liver. In the experiment in which the cholesterol content of multiple organs was measured, the value for the liver and various organs was expressed as mg cholesterol/organ/100 g bw. These values were summed to give whole animal cholesterol content (mg/100 g bw).

Measurement of unesterified and esterified cholesterol content in the liver

In one study involving *npc1*$^{+/+}$ mice fed diets containing variable amounts of cholesterol, aliquots of liver (0.6–0.8 g) were

added to chloroform-methanol (2:1, v/v) and extracted in the presence of two internal standards ([4-^{14}C]cholesteryl oleate and [1,2-^{3}H(N)]cholesterol) (Perkin-Elmer Life Sciences, Inc., Boston, MA). The extracts were filtered into 100 ml volumetric flasks, and a 20 ml aliquot of each was dried under air. The lipids were then dissolved in 2 ml of hexane-tert-butyl methyl ether (100:1.5, v/v) (solvent I) and placed on a silica column (500 mg) (Sep-Pak Vac RC; Waters Corp., Milford, MA) that had been prewashed with 2 ml of solvent I. The column was then eluted with 18 ml of solvent I (to elute esterified cholesterol) and then with 18 ml of tert-butyl methyl ether-glacial acetic acid (100:0.2, v/v) (solvent II) to remove the unesterified cholesterol and other lipids (42). The esterified and unesterified cholesterol fractions were dried under air, saponified in alcoholic KOH, and extracted with petroleum ether. Aliquots of this extract were used to determine the recovery of the radiolabeled standards and also to quantitate the mass of cholesterol by gas chromatography. The unesterified and esterified cholesterol concentrations were added and the proportion (%) of total liver cholesterol in each fraction was calculated.

Measurement of total cholesterol concentration and liver enzyme activities in plasma

Plasma total cholesterol concentration was measured by an enzymatic and colorimetric assay using a reagent mixture from Roche Diagnostics Corp. (catalog No. 11875523; Indianapolis, IN). Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (U/l) were measured by a commercial laboratory.

Determination of biliary bile acid composition

In some of the *npc1*$^{-/-}$ mice that had been fed diets containing added cholesterol (0.20%, w/w) or ezetimibe (0.0125%, w/w) starting at 35 days of age, as well as matching *npc1*$^{-/-}$ and *npc1*$^{+/+}$ mice fed the basal diet alone, bile (usually 5–15 μl) was harvested from the gallbladder and extracted in methanol. The bile acid composition of the extracts was determined by high-pressure liquid chromatography as described (41).

Data analysis

Unless stated otherwise, all data are presented as means ± SEM of values from the specified number of animals. Differences between mean values were tested for statistical significance ($P < 0.05$) using a two-tailed, unpaired Student's *t*-test. Linear regression analysis of data from individual mice was used to determine the correlation between plasma liver enzyme activities and whole liver cholesterol content. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

The main objective of this work was to investigate the extent to which hepatic dysfunction in NPC disease in the mouse correlates with the progressive accumulation of unesterified cholesterol in the liver. In an initial experiment (**Fig. 2**), the plasma levels of ALT and AST and whole liver cholesterol content were measured in large numbers of male and female *npc1*$^{-/-}$ (n = 92) and *npc1*$^{+/+}$ (n = 89) mice over an age span of almost 7 weeks (from 9 to 56 days of age). The data show how hepatic total cholesterol content varied as a function of age in the *npc1*$^{+/+}$ (Fig. 2A) and *npc1*$^{-/-}$ (Fig. 2B) mice during this period of their development. In the case of the *npc1*$^{+/+}$ mice, there was a net increment in hepatic cholesterol content of only 2.5 mg over the 47 day interval. The corresponding value for the *npc1*$^{-/-}$ mice was 32.8 mg. As all mice in this study were fed a basal low-cholesterol, low-fat diet, the increase in the cholesterol content of the liver in the *npc1*$^{+/+}$ mice reflected mostly a growth-related change in the mass of the organ, whereas in the *npc1*$^{-/-}$ mice, the 13-fold greater net change in hepatic cholesterol content primarily reflected endosomal/lysosomal entrapment of sterol. In Fig. 2C, D, the plasma levels of ALT and AST, respectively, are shown as a function of the hepatic cholesterol content in each of the 92 *npc1*$^{-/-}$ animals. The corresponding values for the 89 matching *npc1*$^{+/+}$ mice are presented as the single mean ± SEM. In these two panels, the data demonstrate that in the *npc1*$^{-/-}$ mouse there is a strong positive correlation between plasma ALT and AST activities and the cholesterol content of the whole liver. No such relationship was seen in the matching *npc1*$^{+/+}$ mice. The proportions of unesterified and esterified cholesterol in the livers of these two groups of mice were not determined, because we have shown previously that essentially all of the cholesterol in the liver of *npc1*$^{-/-}$ animals is unesterified (4).

Although there was no evidence of liver cell damage in the *npc1*$^{+/+}$ animals in this study, these results raised the question of whether plasma liver enzyme activities would change significantly in the control mice if their hepatic cholesterol content was increased to the high values typically seen in older *npc1*$^{-/-}$ animals. To explore this possibility, a total of 29 *npc1*$^{+/+}$ mice were fed varying amounts of cholesterol in their diet, up to 1.00%. Several mice were also given a diet with this high level of cholesterol combined with olive oil (10%). The mice were fed their respective diets for 37 days, starting at 19 days of age. As seen in Fig. 2E, F, the plasma activities of ALT and AST, respectively, remained at baseline levels in the face of whole liver cholesterol contents that reached values as high as those that occurred spontaneously in the *npc1*$^{-/-}$ mice. A key point to be made is that in the case of the cholesterol-fed *npc1*$^{+/+}$ mice, almost all of the increase in hepatic cholesterol content occurred in the esterified fraction. This is illustrated in the inset in Fig. 2F. The five individual data points to the far right in Fig. 2E, F represent the mice given the diet containing cholesterol with olive oil. In these five animals, total hepatic cholesterol content averaged 44 ± 3 mg, 88% of which was esterified. In six *npc1*$^{+/+}$ mice that received the basal diet alone, whole liver cholesterol content averaged only 2.8 ± 0.1 mg, of which only 19% was esterified. Thus, this study illustrates the striking difference in toxicity found with the accumulation of unesterified, as opposed to esterified, sterol in the liver cell. Expansion of the amphipathic unesterified cholesterol pool in the late endosomal/lysosomal compartment apparently resulted in significant cell damage, whereas similar accumulation of the very hydrophobic cholesteryl ester pool in the cytosolic compartment caused no cell damage, even at the high level of accumulation of 50 mg/whole liver.

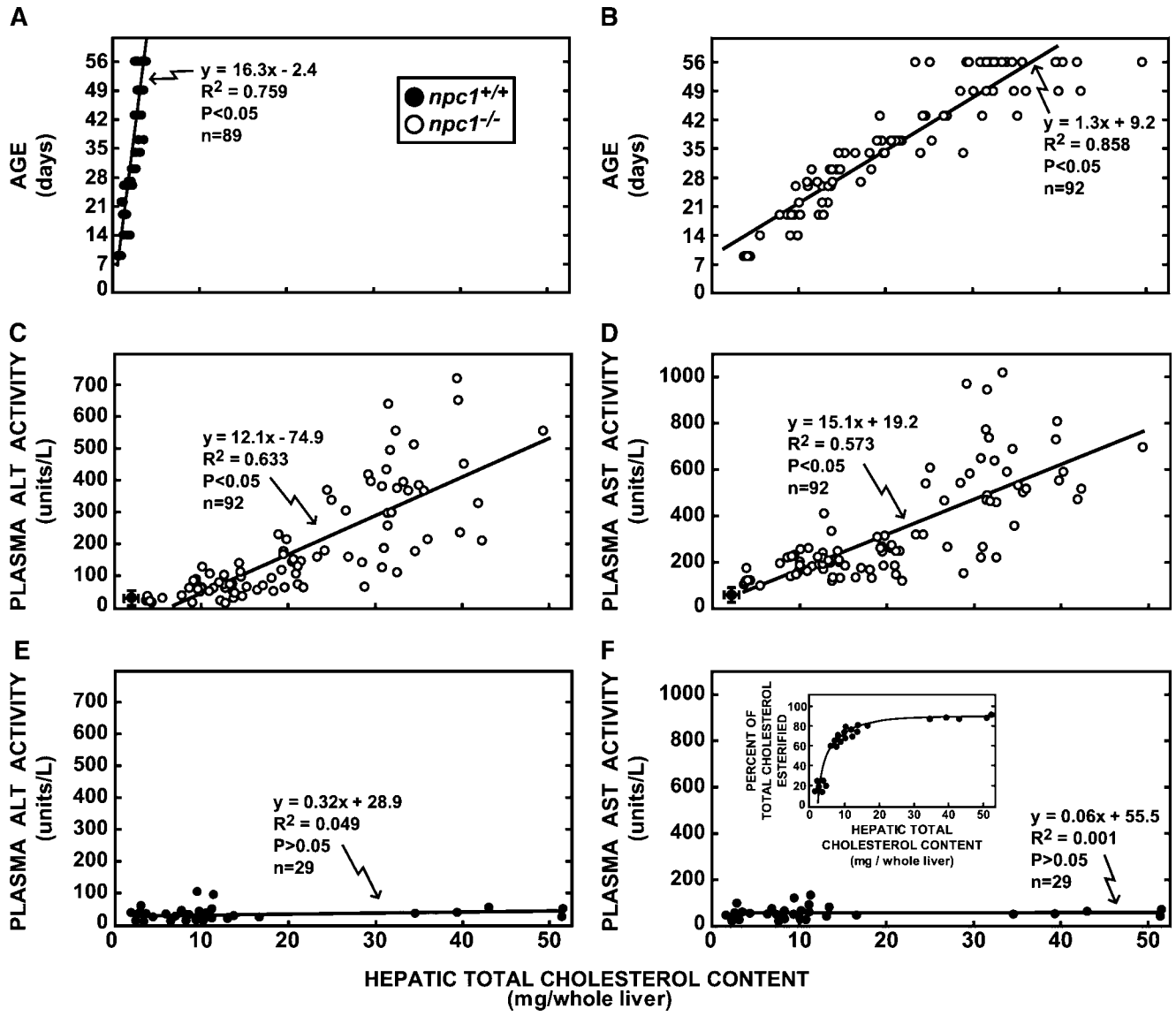

Fig. 2. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as a function of whole liver cholesterol content in *npc1*$^{-/-}$ and *npc1*$^{+/+}$ mice at various ages and in *npc1*$^{+/-}$ mice fed cholesterol-enriched diets. The results of two separate groups of experiments are shown. In the first group (A–D), litters born to *npc1*$^{+/-}$ parents were allowed to suckle until 19 days of age unless studied beforehand. Those pups that were studied before then were genotyped at the time of study. All other pups were weaned and genotyped when 19 days old. These mice were then switched to a basal rodent chow diet with no additions and were studied at various ages up to 56 days. Hepatic cholesterol content and plasma ALT and AST activities were determined. There were approximately equal numbers of male and female *npc1*$^{+/+}$ and *npc1*$^{-/-}$ mice. The data in A and B illustrate the age-related changes in hepatic total cholesterol content in 89 *npc1*$^{+/+}$ (A) and 92 *npc1*$^{-/-}$ (B) mice from 9 to 56 days of age. Each point represents data from an individual animal. The plasma activities of ALT and AST in all 92 *npc1*$^{-/-}$ mice are plotted as a function of hepatic total cholesterol content in C and D, respectively. The corresponding data for the 89 *npc1*$^{+/+}$ mice are presented as single means ± SEM in these panels. The data in A–D were subjected to linear regression analysis, and the resulting respective equations are shown. In the second group of experiments (E, F), *npc1*$^{+/+}$ mice were fed the basal diet alone or diet containing added cholesterol at 0.25, 0.50, or 1.00%. Some mice were fed a diet with 1% cholesterol and 10% olive oil. All mice were female and were fed their respective diets for 37 days starting at 19 days of age. Plasma ALT and AST activities as well as hepatic cholesterol content were then measured. The points to the far right in E and F represent data from five mice fed the diet with cholesterol and olive oil. Each point in E and F represents data from an individual animal (n = 29). These data were also subjected to linear regression analysis, and the resulting respective equations are shown. The inset in F shows the fraction of cholesterol in the liver of all 29 mice that was esterified.

In these studies, both the duration and the magnitude of the expanded pool of unesterified cholesterol varied. In the next set of studies, therefore, the magnitude of the expansion was altered but the duration of the accumulation was kept constant. In this experiment, *npc1*$^{-/-}$ and matching *npc1*$^{+/+}$ mice were fed for 21 days the basal diet containing an ~10- or 50-fold enrichment of cholesterol (i.e., 0.20% and 1.00%, respectively). Data from these mice, which were started on the high-cholesterol diets at 35 days of age, were compared with those from match-

ing groups of *npc1*$^{-/-}$ and *npc1*$^{+/+}$ mice given the basal diet alone.

As shown in **Fig. 3**, on the low (0.02%) cholesterol diet, relative liver weight (A), hepatic total cholesterol content (B), whole animal cholesterol content (C), plasma total cholesterol concentration (D), and transaminase levels (E, F) were all significantly higher in the *npc1*$^{-/-}$ mice compared with the control *npc1*$^{+/+}$ animals. Importantly, however, as the delivery of cholesterol to the liver in the CMr was increased by feeding the 0.20% and 1.00% cholesterol diets, there were further, significant increases in relative liver weight (A), hepatic total cholesterol content (B), whole animal cholesterol content (C), and plasma total cholesterol concentration (D). In particular, there was also a further, significant 3- to 6-fold increase in the plasma transaminase levels (E, F). Thus, under conditions in which the duration of cholesterol feeding was kept constant, the markers of liver cell damage varied directly with the magnitude of the expansion of the pool of unesterified cholesterol in the late endosomal/lysosomal compartment.

The next experiment explored the converse situation. Because cholesterol is delivered continuously to the liver carried in LDL, VLDLr, and CMr (Fig. 1), even on the low-cholesterol (0.02%) diet, the level of hepatic total cholesterol is nearly 10-fold higher in the *npc1*$^{-/-}$ mouse than in the control animal (Fig. 3B). In theory, this expanded pool of unesterified sterol might be substantially reduced

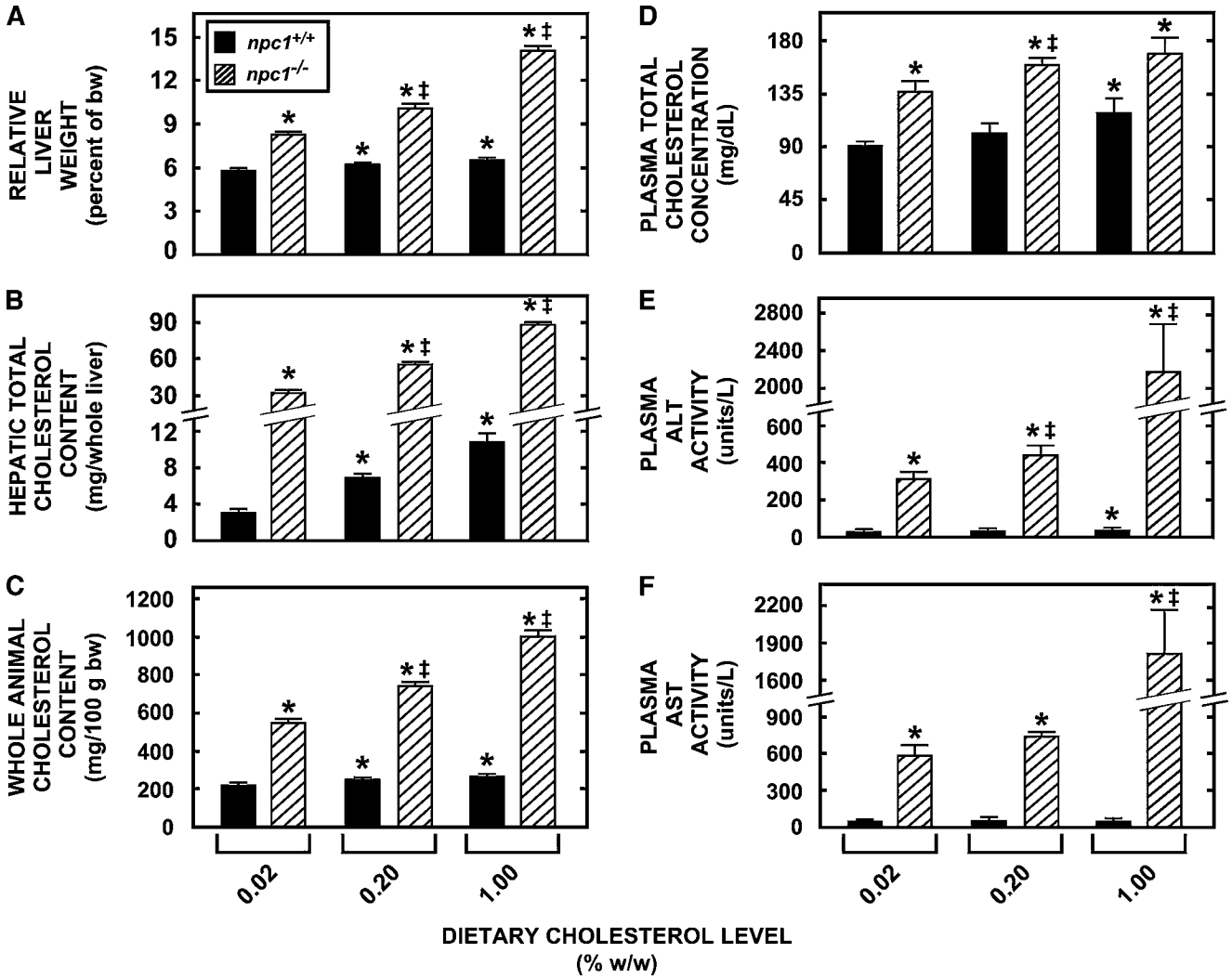

Fig. 3. Effect of varying the dietary cholesterol level on relative liver weight (A), hepatic cholesterol content (B), whole animal cholesterol content (C), plasma cholesterol concentration (D), and plasma levels of ALT (E) and AST (F) in *npc1*$^{-/-}$ and *npc1*$^{+/+}$ mice. All mice were weaned at 19 days of age and thereafter maintained on a basal rodent chow diet until 35 days of age. On that day, six separate experimental groups were established. Matching *npc1*$^{-/-}$ and *npc1*$^{+/+}$ mice were fed one of the following diets for 21 days starting at 35 days of age: one set of mice of each genotype continued to receive only the basal diet (cholesterol level of 0.02%), whereas the second and third matching groups were given the basal diet containing added cholesterol at levels of 0.20% and 1.00%, respectively. All mice were fed their respective diets ad libitum until 56 days old, at which time plasma and liver cholesterol levels and the activities of ALT and AST were determined. There were approximately equal numbers of male and female mice in each group. Values are means ± SEM of data from a minimum of six animals per group for every parameter. * $P < 0.05$ compared with the corresponding value for *npc1*$^{+/+}$ mice fed the basal diet alone; ‡ $P < 0.05$ compared with the corresponding value for *npc1*$^{-/-}$ mice fed the basal diet alone.

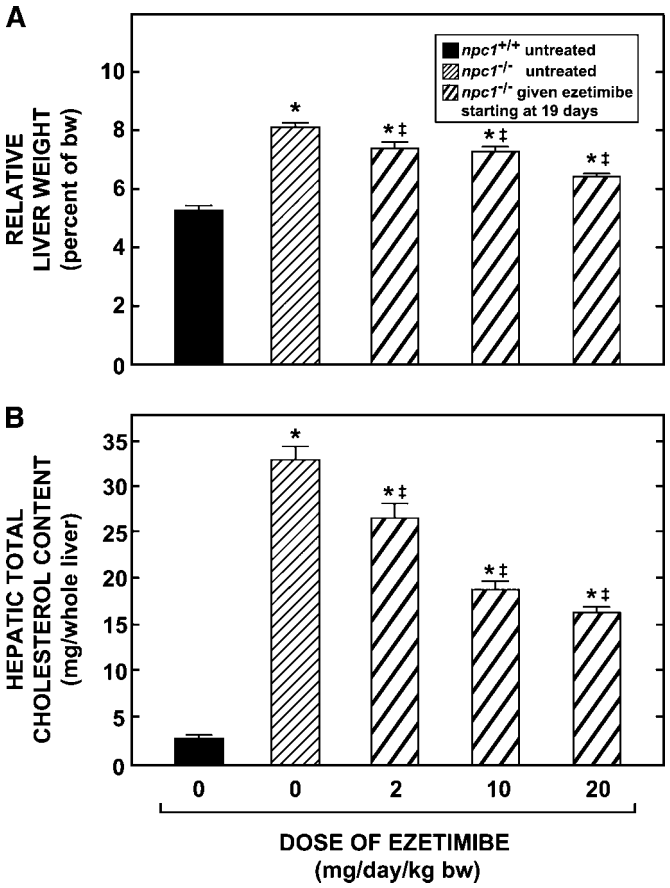

Fig. 4. Relative liver weight (A) and hepatic cholesterol content (B) in $npc1^{-/-}$ mice treated with ezetimibe at various doses. Starting at 19 days of age, three groups of $npc1^{-/-}$ mice were fed the basal diet containing ezetimibe at 0.00125, 0.00625, or 0.0125% [equal to doses of ~2, 10, and 20 mg/day/kg body weight (bw), respectively] until 56 days of age. Matching $npc1^{+/+}$ and $npc1^{-/-}$ mice fed the basal diet alone were run with the groups given ezetimibe. All mice were female. Liver cholesterol content was measured. Values are means ± SEM of data for six mice in each group. * $P < 0.05$ compared with the corresponding value for $npc1^{+/+}$ mice; ‡ $P < 0.05$ compared with the corresponding value for $npc1^{-/-}$ mice fed the basal diet alone.

by blocking the enterohepatic circulation of cholesterol at the step mediated by NPC1L1 (Fig. 1). In the next experiment, therefore, ezetimibe, a potent and selective inhibitor of intestinal sterol absorption, was fed to markedly reduce the delivery of cholesterol carried in CMr to the liver. Ezetimibe was first tested at doses of 2, 10, and 20 mg/day/kg bw in $npc1^{-/-}$ mice starting when they were 19 days old. Their bw gain was not adversely affected at any dose of the drug. However, as shown in **Fig. 4A, B**, there were significant reductions in both relative liver weight and hepatic total cholesterol content, respectively, at all three doses. Although the liver cholesterol content at the 20 mg dose was not statistically different from that at the 10 mg dose ($P > 0.05$), it was decided to use the higher dose in all subsequent experiments.

A separate but related question raised by the studies described in Figs. 3 and 4 was whether there were any genotype-related differences in biliary bile acid composition, and also whether this composition in the mutants changed in response to feeding either cholesterol (0.20%, w/w) or ezetimibe (0.0125%, w/w). As shown by the data in **Table 1**, in the mutant mice there was a shift toward a greater cholic acid enrichment of the pool, and the extent of this was approximately the same whether the $npc1^{-/-}$ mice were given the basal diet alone or the diet containing added cholesterol or ezetimibe. These data also show that, together, cholic and muricholic acids accounted for >90% of bile acids in the pool, irrespective of genotype or dietary treatment.

With this information available, the next complex set of studies explored in detail the relationship between the magnitude and duration of a reduction in the pool of unesterified cholesterol in the cell as well as hepatocellular damage. There were three groups of animals in each of these studies: untreated $npc1^{+/+}$ and $npc1^{-/-}$ animals as well as $npc1^{-/-}$ mice treated with ezetimibe (20 mg/day/kg bw). As shown in **Fig. 5**, hepatic total cholesterol content and plasma activities of ALT and AST were measured in all groups of mice at 56 days of age. In addition, baseline values for these parameters in both $npc1^{+/+}$ and $npc1^{-/-}$ animals were quantitated at 35, 19, and 1 day(s) of age. In all panels of Fig. 5, the dashed line connects the values obtained for the ezetimibe-treated $npc1^{-/-}$ mice at 56 days with the corresponding baseline value for this parameter at 35, 19, or 1 day(s) of age. The solid lines connect the values obtained at 56 days and at baseline for untreated $npc1^{-/-}$ animals and also for matching $npc1^{+/+}$ mice.

When ezetimibe treatment was instituted at 35 days of age, further cholesterol accumulation in the liver was fully

TABLE 1. Biliary bile acid composition in $npc1^{-/-}$ mice given either cholesterol or ezetimibe in their diet

Genotype	Dietary Addition	Number of Animals	Species of Bile Acid in Gallbladder Bile			Ratio of Cholic to Muricholic Acid
			Cholic Acid	Muricholic Acid	Other	
			% of total			
$npc1^{+/+}$	None	6	61.1 ± 0.8	32.5 ± 1.0	6.4 ± 0.6	1.89 ± 0.08
$npc1^{-/-}$	None	4	70.6 ± 0.7[a]	25.9 ± 1.1[a]	3.5 ± 0.8[a]	2.75 ± 0.14[a]
$npc1^{-/-}$	Cholesterol	5	71.8 ± 1.0[a]	25.8 ± 0.5[a]	2.4 ± 0.7[a]	2.79 ± 0.09[a]
$npc1^{-/-}$	Ezetimibe	5	73.9 ± 1.4[a]	23.2 ± 1.3[a]	2.9 ± 0.3[a]	3.23 ± 0.23[a]

NPC1, Niemann-Pick type C1. Female $npc1^{-/-}$ mice were fed a chow diet either with no additions or containing cholesterol (0.2%, w/w) or ezetimibe (0.0125%, w/w) for 21 days starting at 35 days of age. A matching group of $npc1^{+/+}$ mice fed the chow diet with no additions was studied with the $npc1^{-/-}$ animals. Values shown are means ± SEM of data from the number of animals indicated.

[a] $P < 0.05$ compared with the value for $npc1^{+/+}$ mice.

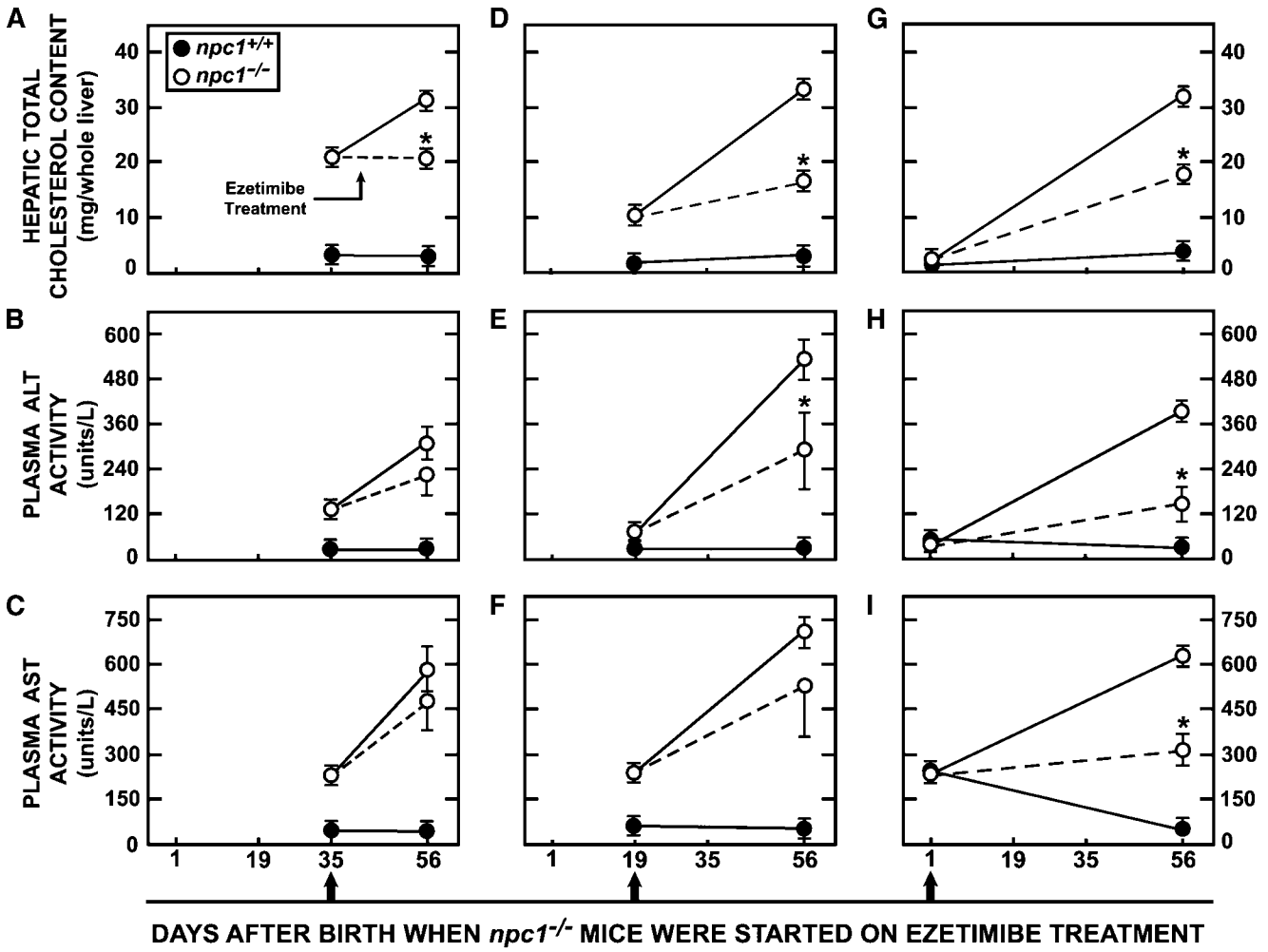

Fig. 5. Hepatic total cholesterol content (A, D, G) and plasma activities of ALT (B, E, H) and AST (C, F, I) in $npc1^{-/-}$ mice given ezetimibe starting at 35 days of age (A–C), 19 days of age (D–F), or from birth (G–I). As described in Materials and Methods, separate groups of $npc1^{-/-}$ mice were started on daily ezetimibe treatment (20 mg/day/kg bw) when they were 35 days old, 19 days old (day of weaning), or ~1 day old, and treatment was continued in all cases until 56 days of age. Matching groups of untreated $npc1^{-/-}$ and $npc1^{+/+}$ mice were studied at 56, 35, 19, and 1 day(s) of age. There were approximately equal numbers of male and female mice in each group except in the case of the treated mice in D–F, which were all female. Values are means ± SEM of data from a minimum of six mice in each group except for the 1 day old animals (G–I), for which there were three or four mice of each genotype. * $P < 0.05$ compared with the corresponding value for the matching group of untreated $npc1^{-/-}$ mice at 56 days.

prevented, so whole liver cholesterol content at 56 days (20.8 ± 0.9 mg) was no different from that when the treatment commenced at 35 days (21.0 ± 0.5 mg) (Fig. 5A). In untreated $npc1^{-/-}$ mice, hepatic cholesterol content reached 31.1 ± 1.6 mg at 56 days. There was also a small attenuation of the increase in plasma activity of both ALT (Fig. 5B) and AST (Fig. 5C) over the treatment period. In matching untreated $npc1^{+/+}$ mice, there was little discernible change in any of these parameters between day 35 and day 56 (Fig. 5A–C). A similar pattern was found when ezetimibe treatment was started earlier at 19 days of age. In this case, however, the level of hepatic total cholesterol content was reduced from 33.0 ± 1.5 mg in the untreated $npc1^{-/-}$ animals to only 17.0 ± 0.7 mg in the ezetimibe-treated group (Fig. 5D), and there was a proportionately greater reduction in the levels of plasma ALT (Fig. 5E) and AST (Fig. 5F).

In the final study, ezetimibe treatment was begun on the day of birth. As shown in Fig. 5G, whole liver cholesterol content in the treated mutants increased from a basal value of 0.8 ± 0.2 mg to only 17.6 ± 1.0 mg at 56 days, less than half the increase found in the untreated mutants over the same period. Commencing ezetimibe treatment at birth also resulted in a more marked blunting of the increase in the plasma activities of both ALT (Fig. 5H) and AST (Fig. 5I) over the same period. Over the same age span of 1 to 56 days, whole liver cholesterol content in matching $npc1^{+/+}$ mice increased by ~3 mg, plasma ALT activity remained low, and plasma AST activity declined from 239 to 42 U/l (Fig. 5G–I). Thus, in contrast to the studies shown in Fig. 3, these experiments clearly demonstrated that the duration over which the expansion of the pool of unesterified cholesterol took place also determined the degree of liver cell damage. In this study,

treatment with ezetimibe resulted in a similar level of hepatic total cholesterol content (17–21 mg/liver) at 56 days of age; however, as the period of treatment was extended from 21 to 37 and, finally, to 56 days, there were reductions in the plasma transaminase levels of ~14, 36, and, finally, 57%, respectively.

Because of the importance of this observation, the profile of this beneficial effect of ezetimibe on hepatic cholesterol levels was explored in more detail in an additional experiment. As shown in **Fig. 6**, both untreated and treated *npc1*$^{-/-}$ mice were followed over the entire 56 day period of observation, and groups of these animals were euthanized at 1, 9, 14, 27, 37, and 56 days of age for the determination of hepatic total cholesterol content. As is evident in Fig. 6A, a cholesterol-lowering benefit of ezetimibe was clearly evident within 9 days of commencing treatment. After this point, there were decisively greater reductions in hepatic cholesterol content in the treated mutants. The plasma activities of ALT and AST in these mice are shown in Fig. 6B and C, respectively. In both cases, these values mirror the differences seen in the cholesterol content of the liver.

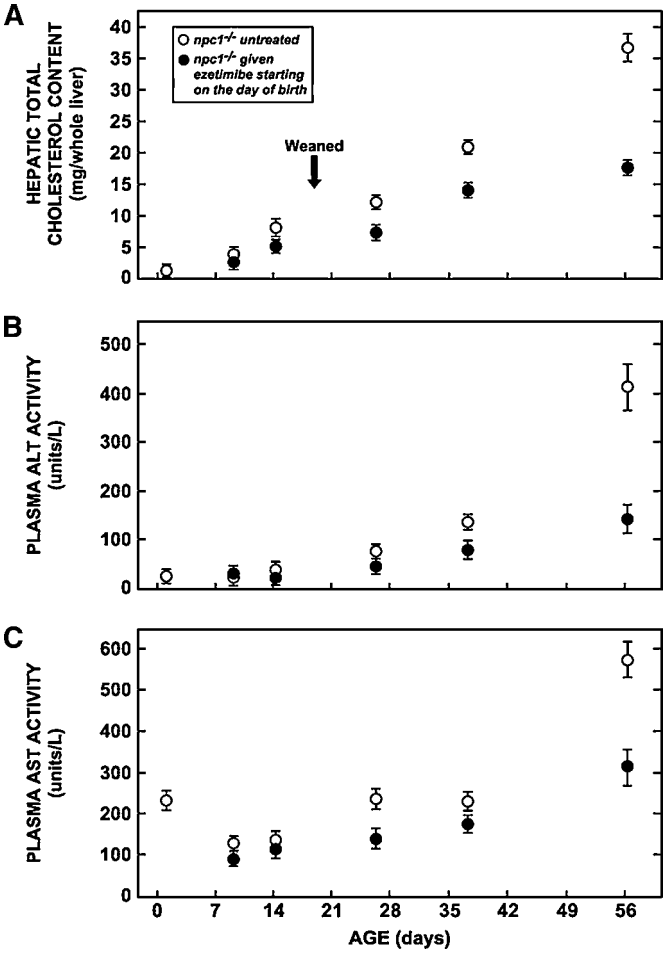

Fig. 6. Hepatic total cholesterol content (A) and plasma ALT (B) and AST (C) activities in *npc1*$^{-/-}$ mice treated with ezetimibe starting on the first day after birth and studied at 9, 14, 27, 37, and 56 days of age. As described in Materials and Methods, separate groups of *npc1*$^{-/-}$ mice were started on ezetimibe treatment (20 mg/day/kg bw) within the first 24 h after birth, and treatment was continued daily until the mice were studied at 9, 14, 27, 37, or 56 days of age. A matching group of untreated *npc1*$^{-/-}$ mice was also studied at each of these ages. There were approximately equal numbers of male and female mice in each group. Values are means ± SEM of data from 6 to 11 mice at each age for all groups except at 1 day (n = 3), 9 days (n = 4 for the treated group), and 14 days (n = 3 for the untreated group).

Together, these various experiments revealed an essentially linear relationship between the level of unesterified cholesterol in the liver and the degree of hepatocyte damage. However, because cholesterol and ezetimibe feeding only affects the amount of sterol reaching the liver in the CMr, there should be no similar effect in any other organ, including the central nervous system. When tested, this prediction proved correct. **Figure 7**, for example, illustrates the effect of ezetimibe administration on the whole organ cholesterol content of different tissues. This drug significantly decreased the content of cholesterol in the liver but had no effect on the content in the spleen, lung, kidney, brain, or remaining organs of the residual carcass. Although blocking cholesterol absorption resulted in a small decrease in whole animal cholesterol content (Fig. 7, inset), this reduction was attributable entirely to the lower pool of sterol in the liver. Finally, because the central nervous system is isolated from the rest of the body by the blood-brain barrier, such a manipulation should not affect the onset of neurodegenerative disease. This prediction also proved to be correct. The administration of ezetimibe from birth did not prolong life (82 ± 3 days vs. 87 ± 4 days) in this mouse model of NPC disease, just as cholesterol feeding did not hasten death (33). Similarly, dietary cholesterol restriction in a feline model of NPC disease did not increase longevity (43). Notably, the matching *npc1*$^{+/+}$ mice given ezetimibe from birth and followed for up to 112 days of age had relative liver weights and plasma transaminase levels that were essentially identical to those in untreated control animals of the same age.

DISCUSSION

These studies provide a detailed description of the relationship between the amount of cholesterol reaching the liver in apoE- and apoB-100-containing lipoproteins and the degree of liver cell damage. In the presence of a mutation in the NPC1 protein, this sterol becomes trapped within the late endosomal/lysosomal compartment of these cells and so cannot be excreted into the bile or used for the turnover of membrane sterol or the synthesis of new lipoproteins and bile acids (4, 30, 44, 45). In these studies, the amount of cholesterol sequestered in this compartment was varied either by interrupting the enterohepatic circulation of sterol at the step in the intestine mediated by NPC1L1 (Fig. 1) or by enriching the chylomicron with excess cholesterol. In this manner, it was possible both to vary the mass of cholesterol trapped in the liver cells from only a few milligrams to nearly 90 mg (Figs. 3, 4) and to alter the duration over which this

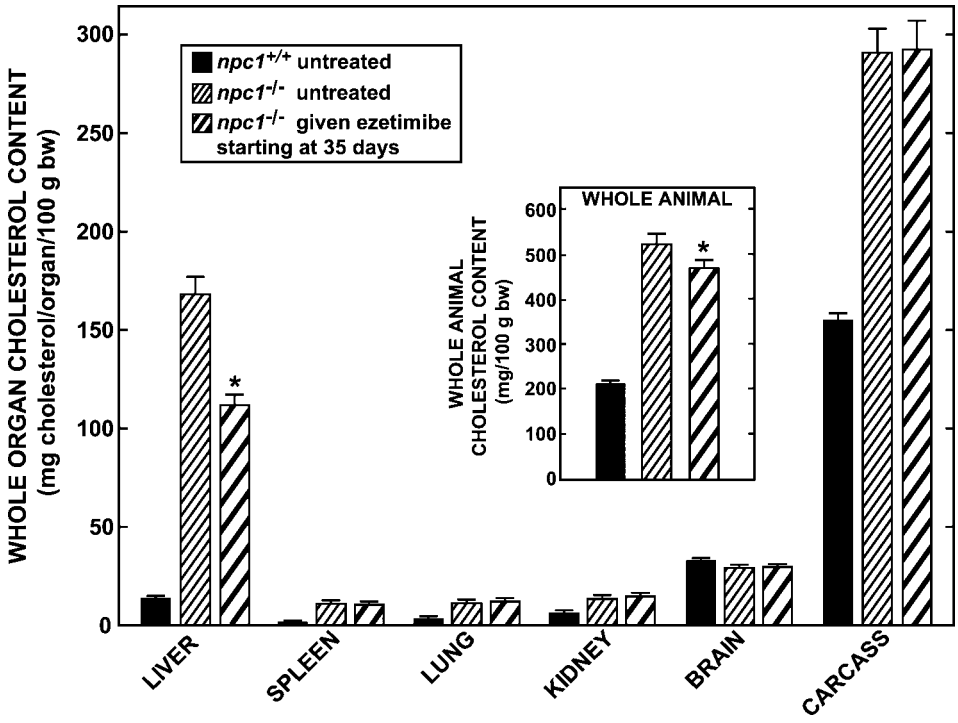

Fig. 7. Whole organ cholesterol content in $npc1^{-/-}$ mice treated with ezetimibe starting at 35 days of age. In some of the mice used in the study described for Fig. 5A–C, cholesterol content was determined in several extrahepatic organs in addition to the liver. The organ cholesterol contents were summed to give whole animal cholesterol content, the values for which are shown in the inset. Values are means ± SEM of data from six untreated $npc1^{+/+}$ mice, 5 untreated $npc1^{-/-}$ mice, and 8 treated $npc1^{-/-}$mice. * $P < 0.05$ compared with the corresponding value for untreated $npc1^{-/-}$ mice.

expansion took place between 1 and 56 days (Fig. 5). Furthermore, these manipulations affected sterol levels specifically in the liver and had no effect on the amount of cholesterol sequestered in the other extrahepatic organs, including the central nervous system (Fig. 7).

Before discussing the significance of the findings in these studies, several points regarding their design warrant emphasis. First, an age of 56 days was chosen as the end point in most experiments because it is just after this time that the mutant mice start to manifest ataxia, consume less food, and begin to lose body weight. Second, the amount of cholesterol in the liver was expressed on a whole organ basis rather than as mg/g of tissue because, as documented previously (31), this provides a more quantitative measure of the degree of cholesterol entrapment in the liver in this model. In this manner, the impact of each experimental manipulation on liver cholesterol homeostasis could be determined more accurately, irrespective of whether a particular treatment affected cellular cholesterol content or the degree of hepatomegaly. Third, the dose of ezetimibe used in most experiments (20 mg/day/kg) was double that shown previously to cause a >90% inhibition of intestinal sterol absorption in the mouse (39). In an animal weighing 20 g, the absolute amount of ezetimibe administered daily at this dose was only ~400 μg. This is only a fraction of the dose at which other agents must be given to significantly block cholesterol absorption (46–48).

The first conclusion drawn from these investigations is that the severity of liver cell damage in this disease is a complex function of both the amount of cholesterol sequestered in the cells and the duration over which this sequestration takes place. Clearly, when the duration was kept constant, the plasma transaminase levels increased as a direct function of the hepatic cholesterol content and reached ~2,000 U/l when the liver content reached ~90 mg of sterol (Fig. 3). However, when the level of sequestration was kept nearly constant, the interval of time over which there was an increase in cellular cholesterol also affected the degree of damage. Thus, the protective effect on plasma transaminase levels brought about by blocking intestinal cholesterol absorption was also proportional to the duration of time the drug was administered. When given for only a short period of time, there was relatively little protection, but when started at birth, there was a nearly 60% reduction in the level of liver cell damage, as judged by plasma transaminase levels (Fig. 5H, I). Unfortunately, further reduction in the hepatic total cholesterol content was not possible, because ezetimibe only blocks the movement of intestinal cholesterol to the liver, whereas cholesterol carried in both VLDLr and LDL continues to be cleared into the hepatocytes (Fig. 1).

The second conclusion from these studies has to do with the nature of the molecule that is responsible for initiating cell damage in this particular lysosomal storage disease. In a related syndrome, Wolman disease, inactivation of lyso-

somal acid lipase leads to an accumulation of cholesteryl ester, not unesterified cholesterol, as well as triacylglycerol in every tissue of the body (49, 50). In the 4 to 8 week old *lal*$^{-/-}$ mouse, for example, the concentration of cholesteryl ester in the liver reached nearly 40 mg/g, which is similar to the level of accumulation of unesterified cholesterol in the liver of the *npc1*$^{-/-}$ animal in these studies (Fig. 6). Importantly, these *lal*$^{-/-}$ mice also had liver dysfunction, as manifest by 11- and 8-fold increases in plasma AST and ALT levels, respectively (Hong Du, personal communication) (49). Thus, these observations suggest that an accumulation of unesterified cholesterol, esterified cholesterol, or triacylglycerol in the late endosomal/lysosomal compartment might initiate cell damage.

In addition, there are at least two other potentially toxic molecules described in NPC disease. In both mouse and human with this syndrome, it was shown that there also is tissue accumulation of sphingomyelin and various glycosphingolipids (37, 51, 52). Furthermore, it was reported that an infant with the NPC mutation and liver disease excreted in its urine several unusual bile acids with a 3β-hydroxy-Δ^5 structure and an oxo or hydroxyl group at the C-7 position (53). This finding may be significant because certain abnormal bile acids are known to be associated with liver disease in newborn infants (54, 55), and some of these may interact with death receptors on hepatocytes to initiate apoptosis (56).

However, of these several potentially toxic molecules, it is possible to exclude a number of them as likely candidates for initiating liver cell damage. As reported previously, the absolute rate of bile acid synthesis in the *npc1*$^{-/-}$ mouse is normal or increased marginally (136 vs. 101 mg/day/kg) (30), and the composition of the biliary pool of acidic sterols is also normal (Table 1). Furthermore, in contrast to the situation in the *lal*$^{-/-}$ animals, the level of hepatic triacylglycerol in the *npc1*$^{-/-}$ mouse is actually reduced markedly (5 vs. 26 mg/whole liver) compared with that in the control animal (31). Although the *lal*$^{-/-}$ mice have liver damage associated with increased levels of cholesteryl esters, apparently sphingomyelin and glycosphingolipids are not increased markedly, at least in humans with this disorder (57). Finally, the experimental methods used to alter the level of sterol in the liver in these studies involved manipulations that were specific to the movement of cholesterol into the hepatocyte and would not have affected the enterohepatic circulation of bile acids or these other molecules. Thus, although this important issue needs further study, it seems likely that it is the accumulation of unesterified cholesterol in the late endosomal/lysosomal compartment that initiates, either directly or indirectly, the liver cell damage that was evident in these studies.

However, this damage may not necessarily depend upon events within the hepatocyte alone. There is accumulating evidence that during this process macrophages are being recruited and activated, and these cells may play a critical role in bringing about parenchymal cell death. In NPC disease, clusters of macrophages are found throughout the liver, spleen, and other organs (31, 32). In the brain, macrophages (i.e., microglia) normally destroy Purkinje cells undergoing programmed cell death during normal brain development (58). In the NPC mouse, these same cells become activated in the adult animal and may play a role in the destruction of subsets of neurons that characteristically die in this syndrome (33, 34, 59). Thus, accumulation of unesterified cholesterol in the late endosomal/lysosomal compartment of the liver cell may not only activate pathways leading to apoptosis but, in addition, may lead to signals that also recruit and activate macrophages that, in turn, can contribute to cell death. The exact nature of these several pathways remains to be elucidated.

Third, these findings emphasize the striking difference in the consequences of accumulating these hydrophobic molecules in the late endosomal/lysosomal compartment compared with the cytosolic compartment. Under physiological conditions, the liver cell normally stores excessive amounts of cholesterol and fatty acid in the cytosolic compartment after conversion of these amphipaths into the very hydrophobic cholesteryl ester and triacylglycerol molecules. Even very large amounts of these nonreactive molecules do not damage the cell (Fig. 2E, F). However, apparently, when unesterified cholesterol accumulates in the late endosomal/lysosomal compartment, a series of events may be initiated that lead to increased mRNA levels for caspace 1, caspace 6, and TNFα and, ultimately, cell death through apoptosis (31).

Finally, these studies suggest several recommendations that might be made for the treatment of newborn infants known to have NPC disease. These infants, as well as mice with this disease (31), commonly manifest prolonged cholestasis, and a subset goes on to develop significant liver disease. The present studies suggest that these abnormalities are related to the load of cholesterol reaching the liver during the neonatal period. The suckling mouse, for example, takes in each day ~54 mg cholesterol/kg bw from its mother's milk under circumstances in which the pup is synthesizing daily in excess of 150 mg/kg sterol (60, 61). In the NPC mouse, this leads to hepatomegaly, increased hepatic cholesterol concentrations, and cholestasis with very high plasma alkaline phosphatase levels (31). The relative cholesterol load is far worse in the breast-fed human infant, which acquires ~18 mg/kg each day of cholesterol from its mother's milk under circumstances in which it is synthesizing only ~25 mg/kg (62, 63). This massive load of exogenous cholesterol relative to the rate of endogenous synthesis in the infant is manifest by the inhibition of endogenous cholesterol synthesis, a nearly 3-fold increase in the circulating LDL-cholesterol concentration, and, in the infant with NPC disease, prolonged cholestasis (62, 64). Given the relationship between dietary cholesterol intake and liver disease in this lysosomal storage disorder, it would seem prudent to avoid breast-feeding and other sources of dietary cholesterol and to begin the administration of an agent such as ezetimibe to the infant as soon as the diagnosis is made. However, although such treatment might ameliorate some of the symptoms of childhood liver disease, it presumably would have no effect on the ultimate development of neurolog-

ical symptoms, because the central nervous system is essentially autonomous with respect to cholesterol turnover (65).

The authors thank Heather Waddell, Brian Griffith, and Thien Tran for their excellent technical assistance and Kerry Foreman for expert preparation of the manuscript. The ezetimibe used in these studies was kindly provided by Dr. Harry R. Davis at the Schering-Plough Research Institute. This work was supported by U.S. Public Health Service Research Grant R01 HL-09610 and Training Grant T32 DK-07745 and by a grant from the Moss Heart Fund.

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