Niemann-Pick C1 expression is not regulated by the amount of cholesterol flowing through cells in the mouse

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Abstract The Niemann-Pick C1 (NPC1) protein functions to regulate the transport of cholesterol from late endosomes/lysosomes to other cellular compartments after lipoprotein uptake through the coated-pit pathway. The present study examines the relative expression of NPC1 mRNA and NPC1 protein in different tissues of the mouse in relation to the uptake of total cholesterol carried in chylomicron remnants (CMr-TC), low density lipoproteins (LDL-TC), cholesteryl ester carried in high density lipoproteins (HDL-CE), and cholesterol synthesis. Results from this study demonstrate that the highest relative expression of NPC1 is in the liver, which is also the tissue with the highest uptake of CMr-TC, LDL-TC, HDL-CE, and cholesterol synthesis. However, there was no similar relation in the remaining tissues. To examine the relative expression of NPC1 in relation to the amount of cholesterol that flowed through the coated-pit pathway, mice were fed a diet supplemented with increasing amounts of cholesterol or cholestyramine. The results from this study demonstrated that there was no relation between the relative expression of NPC1 and the amount of cholesterol that flowed through the coated-pit pathway. We conclude that the relative expression of NPC1 is not regulated by the flow of cholesterol through cells in the mouse and is therefore constitutive.—Garver, W. S., C. Xie, J. J. Repa, S. D. Turley, and J. M. Dietschy. **Niemann-Pick C1 expression is not regulated by the amount of cholesterol flowing through cells in the mouse. *J. Lipid Res.* 2005.** 46: **1745–1754.**

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Niemann-Pick C1 (NPC1) disease is an autosomal recessive lipid storage disorder characterized by hepatosplenomegaly and progressive neurodegeneration (1). The gene responsible for NPC1 disease has been identified in both humans and mice, with the encoded protein being designated the NPC1 protein (2, 3). The predicted amino acid sequence of NPC1 was shown to contain a number of structural motifs that include *i*) a signal peptide at the N terminus for endoplasmic reticulum insertion, *ii*) a highly conserved cysteine-rich region with a leucine zipper referred to as the NPC1 domain, *iii*) five transmembrane domains that represent a sterol-sensing domain homologous to other proteins [HMG-CoA reductase, sterol regulatory element binding protein cleavage-activating protein (SCAP), and Niemann-Pick C1-Like 1 (NPC1L1)] involved in regulating cholesterol metabolism, and *iv*) a dileucine motif at the C terminus that mediates the targeting of proteins to late endosomes/lysosomes (4–8). Mutational analysis of the NPC1 gene has revealed that mutations residing within both the NPC1 domain and the sterol-sensing domain are particularly susceptible to adversely affecting the function of NPC1 and thereby causing NPC1 disease (9–12).

The NPC1 mouse model has proven to be a valuable tool for investigating the function of NPC1 in regulating cholesterol metabolism. Early studies performed using the NPC1 mouse described an accumulation of cholesterol in the liver and a defect in the esterification of lipoprotein-derived cholesterol, in addition to the development of hepatomegaly when these mice were fed a cholesterol-rich diet (13, 14). Subsequent studies determined that the NPC1 mutation results in a time-dependent accumulation of cholesterol in nearly every tissue of the mouse, so that when the NPC1 mouse reaches 7 weeks of age, the whole-body pool of cholesterol is increased by 2.5-fold (~5,400 mg/kg body weight) compared with a normal mouse (~2,200 mg/kg body weight) (15). The one tissue of the NPC1 mouse that seemingly does not ac-

Abbreviations: apoA-I, apolipoprotein A-I; CMr-TC, total cholesterol carried in chylomicron remnants; HDL-CE, cholesteryl ester carried in high density lipoproteins; LDL-TC, total cholesterol carried in low density lipoproteins; LDLR, low density lipoprotein receptor; NPC1, Niemann-Pick C1; NPC1L1, Niemann-Pick C1-Like 1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SR-BI, scavenger receptor class B type 1.

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cumulate cholesterol, the central nervous system, actually also participates in the accumulation of cholesterol. However, unlike the other tissues, progressive neurodegeneration results in a simultaneous loss of myelin cholesterol, which essentially masks the cellular accumulation of cholesterol within cells of the central nervous system (16, 17).

The accumulation of cholesterol in the NPC1 mouse has been shown to result from endocytosis of total cholesterol carried in both chylomicron remnants (CMr-TC) and low density lipoproteins (LDL-TC) through the coated-pit pathway (15, 18). In contrast, studies indicate that cholesterol derived from other sources, such as cholesterol synthesis and uptake of cholesteryl ester carried in high density lipoproteins (HDL-CE) through the scavenger receptor class B type I (SR-BI) pathway, does not contribute to the accumulation of cholesterol in the NPC1 mouse (19). Although endocytosis and processing of CMr-TC and LDL-TC by cells with deficient NPC1 activity are characterized by an accumulation of cholesterol within late endosomes/lysosomes, the rate of cholesterol synthesis in these cells is increased, suggesting an overall defect in the regulation of intracellular cholesterol homeostasis (20–22). More recent studies have determined that cholesterol transport from late endosomes/lysosomes to specialized regions of the Golgi apparatus and plasma membrane for efflux to apolipoprotein A-I (apoA-I) and the formation of HDL are also adversely affected in cells with deficient NPC1 activity (23–26). A schematic representation of the major pathways for the movement of cholesterol through different cells in the body is shown in **Fig. 1**.

Consistent with results indicating a block in the transport of CMr-TC and LDL-TC, studies performed using both human and mouse fibroblasts incubated in the presence of LDL demonstrate that NPC1 is primarily associated with novel late endosomal compartments that transiently interact with cholesterol-enriched late endosomes/lysosomes (27, 28). These novel NPC1-containing compartments have been shown to be multivesicular in nature, to be enriched with lysobisphosphatidic acid, and to contain the cholesterol binding protein MLN64 that is associated with the limiting membrane (28–30). The sterol-sensing domain of NPC1 was recently shown to directly bind cholesterol, suggesting that the NPC1 compartment in general serves as a sterol-modulated lipid-sorting compartment that regulates the retrograde transport of endocytosed cholesterol and glycolipids (31, 32). The transport of endocytosed lipids, which requires NPC1 to have a functional sterol-sensing domain, has been described as a dynamic process involving the rapid migration of NPC1 along microtubules (33, 34).

The present studies were performed to determine the relative expression of NPC1 in relation to the flow of cho-

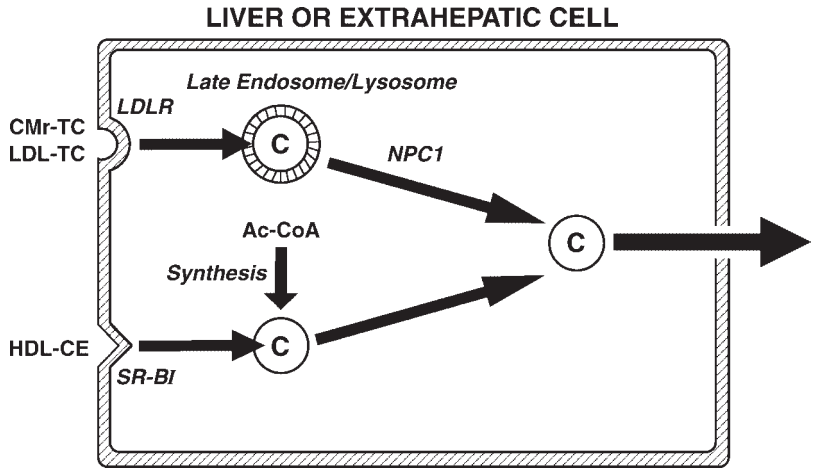

Fig. 1. Major pathways for the movement of cholesterol through different cells in the body. In general, cells continuously acquire cholesterol from de novo cholesterol synthesis and through the uptake of sterol carried in different lipoproteins. In one pathway, total cholesterol carried in chylomicron remnants (CMr-TC) and low density lipoproteins (LDL-TC) is taken up as a result of interaction with the low density lipoprotein receptor (LDLR) that resides on the cell surface. Subsequent clustering of the lipoprotein-receptor complex into coated pits initiates endocytosis and hydrolysis of the cholesteryl ester component of the total cholesterol contained within these particles. The hydrolysis of lipoprotein-derived cholesteryl ester, catalyzed by an acidic cholesteryl ester hydrolase, produces cholesterol that then combines with lipoprotein-derived cholesterol within late endosomes/lysosomes, now designated cholesterol (C). The Niemann-Pick C1 (NPC1) protein facilitates the transport of cholesterol from late endosomes/lysosomes to the metabolically active pool of cholesterol. In the coated-pit pathway, mutation of NPC1 causes an accumulation of cholesterol in late endosomes/lysosomes. A second pathway involves the selective uptake of cholesteryl ester carried in high density lipoproteins (HDL-CE) using scavenger receptor class B type 1 (SR-BI), where the cholesteryl ester is then hydrolyzed in an undefined compartment to produce cholesterol (C). This cholesterol mixes with newly synthesized cholesterol and enters the metabolically active pool of cholesterol destined for excretion from the cell. Mutation of NPC1 does not affect cholesterol flowing through the SR-BI pathway. The magnitude of cholesterol flow through each of these major pathways varies markedly in different cell types throughout organs of the body. Ac-CoA, acetyl-CoA.

lesterol through cells in the mouse. The first experiment was performed to determine the relative expression of NPC1 protein in livers and cerebrums of mice from different genetic backgrounds. The results indicate that the relative expression of NPC1 protein in these tissues was approximately the same. As a result, the BALB/c mouse strain was used throughout the remainder of the study to conduct additional experiments. The second two sets of experiments measured the relative expression of NPC1 mRNA and NPC1 protein in the various tissues of the mouse. The relative expression of NPC1 protein in these tissues, including five regions of the central nervous system, were compared with the combined rates of CMr-TC and LDL-TC uptake through the coated-pit pathway and with the combined rates of cholesterol synthesis and HDL-CE uptake through the SR-BI pathway. The relative expression of NPC1 mRNA and NPC1 protein were then determined in relation to the flow of cholesterol through the coated-pit pathway by supplementing diets with increasing amounts of cholesterol or cholestyramine. Together, the results obtained from this study indicate that although the relative expression of NPC1 mRNA and NPC1 protein varied markedly among various tissues of the mouse, the flow of cholesterol through the cells in the mouse does not regulate the relative expression of NPC1.

MATERIALS AND METHODS

Animals

The Institute Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center approved the studies described in this report. The BALB/c mouse that was primarily used in this study was generated from an inbred colony. Other mice from different genetic backgrounds (DBA, C57BL/6, FVB/NJ, and 129/Sv) that were used in one experiment were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in plastic colony cages in rooms with alternating 12 h periods of light and dark. Most mice were fed ad libitum and maintained on a low-cholesterol (0.016%, w/w) basal rodent diet (No. 7001; Harlan Teklad, Madison, WI). In one study, mice were fed ad libitum a low-cholesterol basal rodent diet supplemented with 0.1% cholesterol, 1% cholesterol, or 2% cholestyramine. All studies were performed on mice at 7 weeks of age in the fed state at the mid-dark phase of the light cycle.

Isolation and radiolabeling of LDL and HDL

Mouse plasma was harvested from both male and female NPC1$^{+/+}$/low density lipoprotein receptor-deficient (LDLR$^{-/-}$) mice maintained on a low-cholesterol basal rodent diet (35). The LDL and HDL fractions were isolated using preparative ultracentrifugation in the density ranges of 1.020–1.055 g/ml and 1.070–1.210 g/ml, respectively. The LDL fraction was radiolabeled with either ^{125}I-labeled tyramine cellobiose or ^{131}I, followed by passage through a heparin-Sepharose CL-6B column (Pharmacia Biotech, Uppsala, Sweden) to remove any contaminating apoE-containing HDL (35, 36). The apoB moiety of LDL was radiolabeled with ^{125}I-labeled tyramine cellobiose using an established technique that allows this radiolabel to enter cells and become trapped within late endosomes/lysosomes after apoB has been degraded, thereby serving as a cumulative measure for the uptake of LDL (37, 38). The radiolabeled LDL preparation was then dialyzed and passed through a 0.45 μm pore size Millex-HA filter before injection. The HDL fraction was radiolabeled with [1α,2α,(*n*)-^{3}H]cholesteryl oleyl ether and [4-^{14}C]cholesteryl oleate by exchange from donor liposomes (39–41). The HDL particle was radiolabeled with [1α,2α,(*n*)-^{3}H]cholesteryl oleyl ether using an established technique that allows this radiolabel to enter cells and become trapped, thereby serving as a cumulative measure for cholesteryl ester uptake from HDL (42). Freshly collected rabbit plasma was used as the source of cholesteryl ester transfer protein, in which rabbit HDL accounted for <5% of the HDL contained in the reaction mixture. The radiolabled HDL was reisolated by ultracentrifugation in the density range of 1.070–1.210 g/ml and dialyzed against isotonic saline. Both radiolabled LDL and HDL fractions were used within 48 h of preparation.

Measurement of LDL and HDL clearance rates in vivo

The mice were anesthetized at the mid-dark phase of the light cycle with diethyl ether and xylazine, and a catheter was inserted into the jugular vein. After awakening, the mice were administered a bolus of ^{125}I-labeled tyramine cellobiose-labeled LDL followed by a continuous infusion of the same preparation for 4 h at a rate determined to maintain a constant specific activity within the plasma (19, 35). Exactly 10 min before termination of the 4 h infusion, a bolus of ^{131}I-labeled LDL was administered through the same catheter. After infusion of the second bolus for 10 min, the mice were anesthetized and exsanguinated, the tissues were removed, rinsed, and weighed, and the amount of ^{125}I and ^{131}I associated with each tissue was determined. An identical procedure was used for the radiolabeled HDL preparations. In both cases, the data from the 10 min infusion were used to correct tissue samples for contaminating blood, whereas the steady-state specific activity of the LDL and HDL fractions obtained from the 4 h infusion was used to calculate the clearance rates of sterol in these fractions (43, 44). The rates of LDL-TC and HDL-CE uptake by the tissues were calculated by multiplying these rates for the clearance of LDL and HDL by the plasma concentration of LDL-TC and HDL-CE and then expressing these values as micrograms of lipoprotein cholesterol taken up each hour per gram of tissue.

Measurement of cholesterol synthesis rates in vivo

The rates of cholesterol synthesis for each tissue were measured in vivo at the mid-dark phase of the light cycle. The mice were injected intraperitoneally with ~50 mCi of [^{3}H]water contained in 100 μl of isotonic saline solution (45–47). One hour later, the mice were anesthetized and exsanguinated, and the tissues were removed. These tissues and the remaining carcass were saponified, and the digitonin-precipitable sterols were isolated (45, 46). The rates of sterol synthesis in each tissue were calculated and expressed as nanomoles of [^{3}H]water incorporated into sterol per hour per gram of tissue. The rate of incorporation of [^{3}H]water into sterols by tissues was converted to an equivalent milligram quantity of cholesterol, assuming that 0.69 ^{3}H atoms were incorporated into the sterol molecule per carbon atom entering the biosynthetic pathway as acetyl-CoA (45, 46). These rates were then expressed as micrograms of cholesterol synthesized each hour per gram of tissue.

Measurement of intestinal cholesterol absorption

Cholesterol absorption was measured by a modified fecal dual-isotope ratio method using [4-^{14}C]cholesterol and [5,6-^{3}H]sitostanol (stigmastanol) (48, 49). The mice were dosed toward the end of the dark phase of the light cycle, and stools were collected from each animal over a 3 day period immediately after dosing

with the radiolabeled sterols contained in medium chain triacylglycerol oil (Mead Johnson and Co., Evansville, IN). The medium chain triacylglycerol oil was used as a dosing vehicle for these studies because its constituent fatty acids have an essentially neutral effect upon cholesterol metabolism and also because medium chain triacylglycerol oil yields a reproducible and consistent result (50). Aliquots of ground stool and the dosing mixture were extracted, and the ratio of ^{14}C and ^{3}H was determined. These data were then used to calculate the percentage of dietary cholesterol that was absorbed. This percentage value multiplied by the daily cholesterol intake gave the absolute rate of sterol absorption per day. These data were used to calculate the amount of dietary cholesterol absorbed each hour and delivered to the liver (micrograms per hour per gram of tissue). The CMr-TC was calculated from dietary cholesterol concentration, consumption of mouse food, and cholesterol absorption.

Northern blot analysis

Total RNA was obtained from mouse tissue using RNA STAT60 (Tel-Test, Inc., Friendswood, TX), and the mRNA was isolated using oligo(dT)-cellulose columns (Pharmacia Biotech). The isolated mRNA was prepared by pooling equal quantities of total hepatic RNA from six mice per treatment group; 5 μg/lane was size-fractionated on 1% formaldehyde agarose gels and transferred to Zetaprobe nylon membranes (Bio-Rad Laboratories, Hercules, CA). A cDNA fragment of mouse NPC1 was generated by RT-PCR using the following primers (NPC1-F, 5′-GGTGCTGGACAGCCAAGTA; NPC1-R, 5′-TCAGCCAGTCCTTCAGCAG) along with mouse brain total RNA as a template. The resulting PCR product contained nucleotides 1,605–2,629 of the mouse NPC1 sequence (GenBank accession number NM_008720). Rat cyclophilin cDNA was kindly provided by David Russell (University of Texas Southwestern Medical Center). The ^{32}P-labeled cDNA probes were prepared using the Rediprime II Random Prime labeling system, and unincorporated ^{32}P-dCTP was removed with a G50 spin column. The Northern blot membranes were hybridized with the radiolabeled probes, and the NPC1 bands (5.8 kb) were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) normalized against cyclophilin.

Tissue homogenization and protein determination

Mouse tissues were homogenized in ice-cold hypotonic homogenization buffer containing 10 mM sodium phosphate, pH 7.4, and a complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) using a motor-driven Teflon and glass homogenizer. A fraction of the tissue homogenate was removed and diluted into three parts ice-cold solubilization buffer containing 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 10 mM SDS, and a complete protease inhibitor cocktail. This solubilization mixture was placed onto a rotating rack in the cold room and allowed to mix for a period of 30 min. In preparation for immunoblot analysis, the concentration of solubilized protein was determined using the bicinchoninic acid protein assay method (Pierce-Endogen, Rockford, IL) with BSA as a standard.

Immunoblot analysis

An equivalent amount of solubilized protein (50 μg) from each sample was separated using 6.0% SDS-PAGE under reduced conditions and transferred to a nitrocellulose membrane (51). Immunoblot buffer containing 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk was used to block nonspecific sites on the membrane for a period of 2 h. The membrane was then incubated in the presence of immunoblot buffer containing NPC1 antibody in the cold room while mixing overnight (28). The next day, the membrane was rinsed with immunoblot buffer (3 × 10 min) and incubated in the presence of immunoblot buffer containing peroxidase-conjugated anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for a period of 1 h. Finally, the membrane was rinsed with immunoblot buffer (3 × 10 min) and ECL was performed using SuperSignal Substrate for Western Blotting (Pierce-Indogen) with images obtained on BioMax MR film (Eastman Kodak, New Haven, CT). The amount of NPC1 protein was quantitated within the linear range of the film using a Bio-Rad model GS-700 Imaging Densitometer.

Calculations

Quantitative data are represented as mean values ± SEM of four to six mice. The two-tailed, unpaired Student's *t*-test was used to determine statistically significant differences ($P < 0.05$) between mean values.

RESULTS

Relative expression of NPC1 protein in the liver and cerebrum of mice from different genetic backgrounds

It is well recognized that there are marked differences in the metabolism of cholesterol by different strains of mice (52, 53). Using immunoblot analysis to measure the amount of NPC1 protein, a study was performed to determine the relative expression of NPC1 protein in livers and cerebrums of mice from six different genetic backgrounds (BALB/c, DBA, C57BL/6, FVB/NJ, mixed, and 129/Sv) (**Fig. 2**). The results indicate that although there were sig-

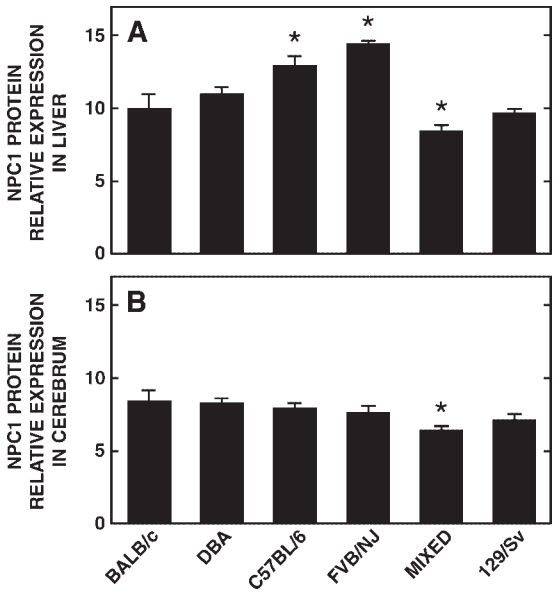

Fig. 2. Relative expression of NPC1 protein in the liver and cerebrum of mice from different genetic backgrounds. A nearly equal number of male and female mice from six different genetic backgrounds (BALB/c, DBA, C57BL/6, FVB/NJ, mixed, and 129/Sv) were maintained on a low-cholesterol (0.016%, w/w) basal rodent diet. The relative expression of NPC1 protein was determined in liver (A) and cerebrum (B) of these mice at 7 weeks of age. Each value represents the mean ± SEM for four to six mice in each group. The relative expression of NPC1 protein in the liver of BALB/c mice was arbitrarily set to a value of 10. Asterisks indicate a statistically significant difference ($P < 0.05$) between mean values compared with BALB/c mice.

nificant differences in the relative expression of NPC1 protein in livers and cerebrums between some mice of different genetic backgrounds, overall, the relative expression of NPC1 protein in these tissues was similar. With respect to livers, the largest difference in the relative mean expression of NPC1 protein occurred between FVB/NJ and mixed mice, where the FVB/NJ mouse livers expressed ~40% more NPC1 protein than the mixed mouse livers (Fig. 2A). With respect to cerebrums, the largest difference in the relative mean expression of NPC1 protein occurred between BALB/c and mixed mice, where the BALB/c mouse cerebrums expressed ~25% more NPC1 protein than the mixed mouse cerebrums (Fig. 2B). Because the relative expression of NPC1 protein in livers and cerebrums from BALB/c mice were approximately the same between most mice of the different genetic backgrounds, the remaining studies were performed using only the BALB/c strain of mouse.

Relative expression of NPC1 mRNA in different tissues of the mouse

It has been shown that NPC1 mice accumulate excess cholesterol in every tissue, including cells of the central nervous system (15, 16, 18). In this study, the relative expression of NPC1 mRNA in the different tissues of the mouse was determined using Northern blot analysis (**Fig. 3**). The relative expression of cyclophilin mRNA was also determined and used as a loading control for total mRNA. The relative expression of NPC1 mRNA in the different tissues of the mouse could be categorized into three groups representing high, medium, and low relative expression. With the relative expression of NPC1 mRNA in the liver being arbitrarily set to a value of 10, the results indicate that a high relative expression of NPC1 mRNA (values > 5) was present in adrenals, eye, liver, lung, and placenta. A medium relative expression of NPC1 mRNA (values between 2.5 and 5) was present in brown adipose, brain, heart, ovary, skin, and uterus. A low relative expression of NPC1 mRNA (values < 2.5) was present in white adipose, colon, small intestine, kidney, skeletal muscle, spleen, and testes. These results demonstrate that although NPC1 mRNA is widely expressed in different tissues of the mouse, the relative expression of NPC1 mRNA among the different tissues of the mouse varies.

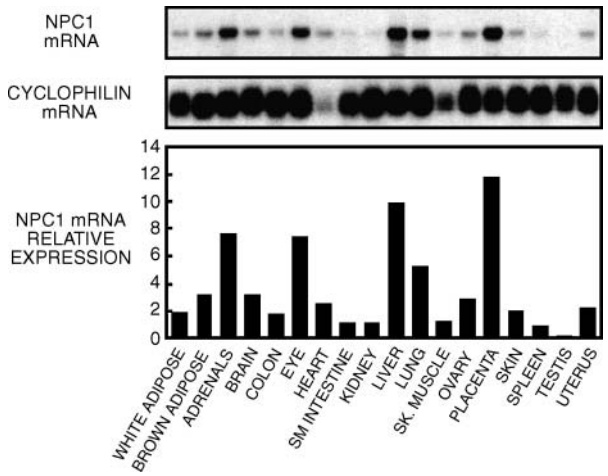

Fig. 3. Relative expression of NPC1 mRNA in different tissues of the mouse. Northern blot analysis for NPC1 mRNA and cyclophilin mRNA was performed on different tissues pooled from mice that were 7 weeks old and maintained on a low-cholesterol (0.016%, w/w) basal rodent diet. The relative expression of NPC1 mRNA and cyclophilin mRNA was determined using an equivalent amount of isolated total mRNA prepared from different tissues of the mouse. The relative expression of NPC1 mRNA and cyclophilin mRNA in different tissues of the mouse was quantitated, and the relative expression of NPC1 mRNA in the liver was arbitrarily set to a value of 10. SK., skeletal; SM., small.

Relative expression of NPC1 protein in relation to the rate of lipoprotein cholesterol uptake and cholesterol synthesis in different tissues of the mouse

Although the last study demonstrated that NPC1 mRNA was expressed in nearly all tissues, the next study was performed to determine the relative expression of NPC1 protein in relation to the rate of cholesterol flowing through the two major pathways described in Fig. 1 (**Fig. 4**). In this study, the relative expression of NPC1 protein was determined using nine different tissues (Fig. 4A), including five different regions of the central nervous system (Fig. 4B). Similar to results obtained measuring the relative expression of NPC1 mRNA, the relative expression of NPC1 protein in the different tissues of the mouse could be categorized into three groups representing high, medium, and low relative expression. With the relative expression of NPC1 protein in the liver being arbitrarily set to a value of 10, the results indicate that a high relative expression of NPC1 protein (values > 5) was present in liver, lung, and cerebrum. A medium relative expression of NPC1 protein (values between 2.5 and 5) was present in spleen, colon, heart, cerebellum, mid-brain, brain stem, and spinal cord. A low relative expression of NPC1 protein (values < 2.5) was present in kidney, stomach, small intestine, and skeletal muscle. Similar to the expression of NPC1 mRNA measured in different tissues of the mouse, these results demonstrate that NPC1 protein is widely expressed in different tissues of the mouse but that the relative expression of NPC1 protein among the different tissues varies. The first major pathway for cholesterol flowing through these various tissues is represented by the uptake of CMr-TC and LDL-TC through the coated-pit pathway using the LDLR (Fig. 1). To determine the magnitude of cholesterol flowing through this pathway, the rates of cholesterol absorption from the intestine and of clearance for LDL-TC into each tissue were quantitated. The results of these two measurements were combined and expressed as micrograms of cholesterol passing through the coated-pit pathway each hour per gram of each tissue. As the results indicate, the vast majority of CMr-TC and LDL-TC uptake occurred in the liver (Fig. 4C), whereas there was little or no detectable uptake of CMr-TC and LDL-TC in other organs (Fig. 4C), particularly in regions of the central nervous system (Fig. 4D). The second major pathway for cholesterol flowing through these cells represents the selective uptake of HDL-CE using SR-BI and de novo cholesterol synthesis

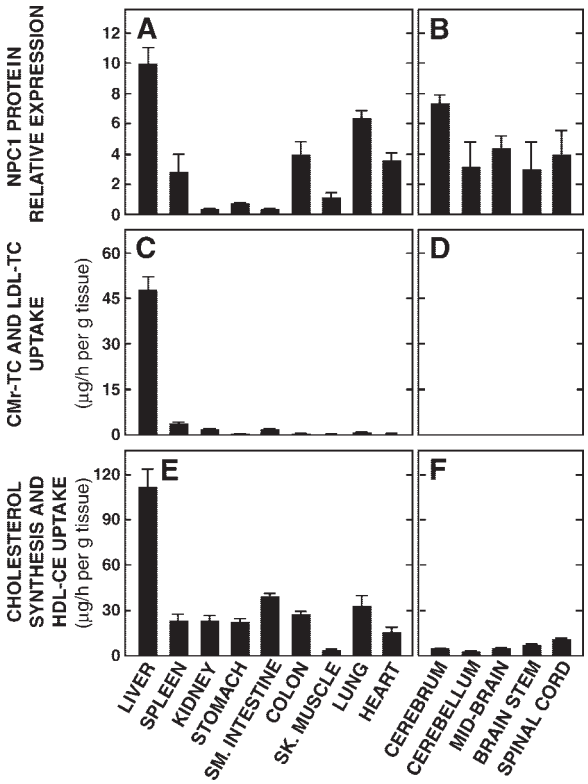

Fig. 4. Relative expression of NPC1 protein in relation to the rate of lipoprotein cholesterol uptake and cholesterol synthesis in different tissues of the mouse. A nearly equal number of male and female mice were maintained on a low-cholesterol (0.016%, w/w) basal rodent diet. The relative expression of NPC1 protein was determined using an equivalent amount of protein from different tissues of mice at 7 weeks of age. The relative expression of NPC1 protein was determined in nine different tissues (A) and five different regions of the central nervous system (B). The relative expression of NPC1 protein in these different tissues is shown in relation to the combined uptake of CMr-TC and LDL-TC (C, D) through the coated-pit pathway and the combined cholesterol synthesis and uptake of HDL-CE (E, F) as described for Fig. 1. The values shown for the flow of cholesterol through the two pathways are represented as micrograms of cholesterol flowing through the cells per hour per gram of tissue. Each value represents the mean ± SEM for four to six mice. The relative expression of NPC1 protein in the liver was arbitrarily set to a value of 10. SK., skeletal; SM., small.

(Fig. 1). Again, the rates for both of these processes were quantitated and then combined. As expected, the major site for the selective uptake of HDL-CE and cholesterol synthesis was the liver (Fig. 4E), whereas the remaining tissues (Fig. 4E), including regions of the central nervous system (Fig. 4F), had lower rates of selective uptake and cholesterol synthesis. Therefore, these results clearly demonstrate that the highest flow of cholesterol through the coated-pit pathway was in the liver (Fig. 4C), whereas the flow of cholesterol through the coated-pit pathway was much lower or absent in the remaining tissues (Fig. 4C), including the central nervous system (Fig. 4D). Nevertheless, the NPC1 protein was substantially expressed in nearly all tissues (Fig. 4A), including regions of the central nervous system (Fig. 4B), which correlated poorly, if at all, with cholesterol flowing through the coated-pit pathway in these tissues.

Relative expression of NPC1 mRNA in relation to the flow of cholesterol through the coated-pit pathway in the liver

To determine whether the relative expression of NPC1 mRNA is regulated by the flow of cholesterol through the coated-pit pathway of the liver, mice were fed diets supplemented with increasing amounts of either cholesterol or cholestyramine (**Fig. 5**). At 7 weeks of age, one group of mice was fed a low-cholesterol basal rodent diet, whereas the other groups were fed a low-cholesterol basal rodent diet supplemented with either 0.1% or 1% cholesterol or a low-cholesterol basal rodent diet supplemented with 2% cholestyramine for a period of 1 week. The results indicate that after feeding mice for 1 week a low-cholesterol basal rodent diet, a low-cholesterol basal rodent diet supplemented with either 0.1% or 1% cholesterol, or a low-cholesterol basal rodent diet supplemented with 2% cholestyramine, the respective hepatic cholesterol concentrations were 2.3 ± 0.05, 3.9 ± 0.10, 9.9 ± 1.56, or 2.3 ± 0.06 mg cholesterol/g tissue. Therefore, the supplementation of cholesterol and cholestyramine into diets increased the flow of cholesterol through the coated-pit pathway in the liver. Northern blot analysis was used to determine the relative expression of NPC1 mRNA and cyclophilin mRNA, with the amount of cyclophilin mRNA being used as a loading control to normalize for the amount of NPC1 mRNA. The results indicate that there was no significant

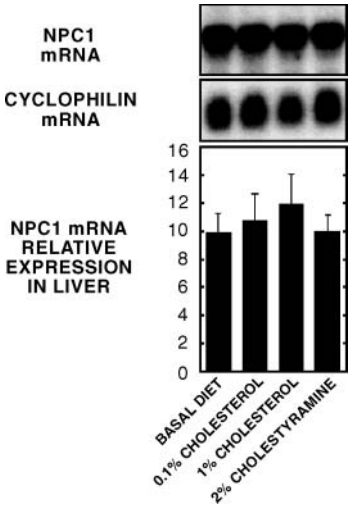

Fig. 5. Relative expression of NPC1 mRNA in relation to the flow of cholesterol through the coated-pit pathway in the liver. A nearly equal number of male and female mice were maintained on a low-cholesterol (0.016%, w/w) basal rodent diet. At 7 weeks of age, one group of mice was fed the same low-cholesterol basal rodent diet, whereas other groups were fed a low-cholesterol basal rodent diet supplemented with either 0.1% or 1% cholesterol or a low-cholesterol basal rodent diet supplemented with 2% cholestyramine for a period of 1 week. Northern blot analysis for NPC1 mRNA and cyclophilin mRNA was then performed on the livers. The relative expression of NPC1 mRNA and cyclophilin mRNA was determined using an equivalent amount of isolated total mRNA prepared from the livers. Each value represents the mean ± SEM for four to six mice. The relative expression of NPC1 mRNA in the livers of the animals fed the low-cholesterol basal rodent diet was arbitrarily set to a value of 10.

difference in the relative expression of NPC1 mRNA in relation to the amount of cholesterol or cholestyramine supplemented in the diets, indicating that the flow of cholesterol through the coated-pit pathway in the liver, as a result of the uptake of CMr-TC and LDL-TC, does not have an affect upon the relative expression of NPC1 mRNA.

Relative expression of NPC1 protein in relation to the flow of cholesterol through the coated-pit pathway in the liver and cerebrum

To determine whether the relative expression of NPC1 protein is regulated by the flow of cholesterol through the coated-pit pathway of the liver and cerebrum, mice were fed diets supplemented with increasing amounts of either cholesterol or cholestyramine (**Fig. 6**). At 7 weeks of age, one group of mice was fed a low-cholesterol basal rodent diet, whereas the other groups were fed a low-cholesterol basal rodent diet supplemented with either 0.1% or 1% cholesterol or a low-cholesterol basal rodent diet supplemented with 2% cholestyramine for a period of 1 week. Using immunoblot analysis, the relative expression of NPC1 protein was determined in mouse livers and cerebrums. Consistent with the results obtained for NPC1 mRNA, the results indicate that there is no significant difference in the relative expression of NPC1 protein in relation to the amount of cholesterol or cholestyramine supplemented in the diets, suggesting that the flow of cholesterol through the coated-pit pathway in the liver and cerebrum, as a result of the uptake of CMr-TC and LDL-TC, does not have an affect upon the relative expression of NPC1 protein.

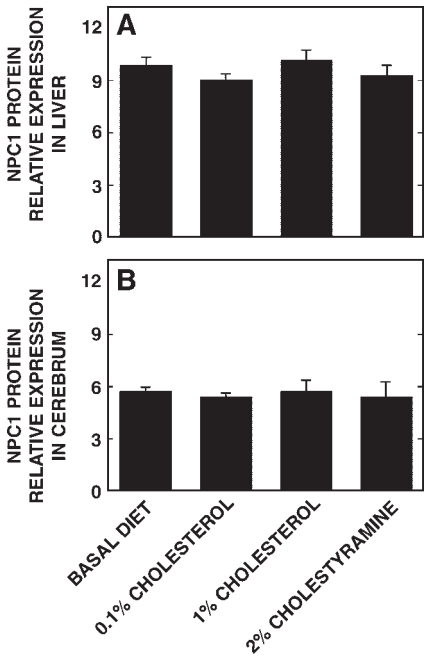

Fig. 6. Relative expression of NPC1 protein in relation to the flow of cholesterol through the coated-pit pathway in the liver and cerebrum. A nearly equal number of male and female mice were maintained on a low-cholesterol (0.016%, w/w) basal rodent diet. At 7 weeks of age, one group of mice was fed the same low-cholesterol basal rodent diet, whereas other groups were fed a low-cholesterol basal rodent diet supplemented with either 0.1% or 1% cholesterol or a low-cholesterol basal rodent diet supplemented with 2% cholestyramine for a period of 1 week. Immunoblot analysis for NPC1 protein was then performed on the livers and cerebrum. The relative expression of NPC1 protein was determined using an equivalent amount of protein prepared from livers and cerebrums. Each value represents the mean ± SEM for four to six mice. The relative expression of NPC1 protein in the liver of the animals fed the low-cholesterol basal rodent diet was arbitrarily set to a value of 10.

DISCUSSION

These studies provide the first results describing *i*) the relative expression of NPC1 protein in the liver and cerebrum of mice from different genetic backgrounds, *ii*) the relative expression of NPC1 mRNA and NPC1 protein in different tissues of the mouse, *iii*) the relative expression of NPC1 protein in relation to the rates of CMr-TC, LDL-TC, and HDL-CE uptake and cholesterol synthesis in different tissues of the mouse, and *iv*) the relative expression of NPC1 mRNA and NPC1 protein in relation to the flow of cholesterol through the coated-pit pathway of the liver and cerebrum. Together, the results obtained from this study indicate that although the relative expression of NPC1 mRNA and NPC1 protein vary markedly among different tissues of the mouse, the flow of cholesterol through the cells in the mouse does not regulate the relative expression of NPC1; therefore, NPC1 is expressed constitutively.

It has been shown that mice from different genetic backgrounds differ with respect to lipoprotein metabolism, expression of plasma apolipoproteins, responsiveness to high-fat and high-cholesterol diets, and susceptibility to aortic atherosclerosis as a result of altered gene expression and naturally occurring polymorphisms (52, 54–56). For instance, one study demonstrated marked variations in the concentrations of plasma cholesterol and triglyceride among mice of different genetic backgrounds, reportedly attributable to differences in cholesterol absorption and bile acid synthesis (52). In the present study, although the results indicate that there are significant differences in the relative expression of NPC1 protein in livers and cerebrums between certain mice of different genetic backgrounds, overall, the relative expression of NPC1 protein in these tissues was similar and did not vary by greater than 40% in the livers and 25% in the cerebrums. Interestingly, consistent with the ability of certain mouse strains to possibly have differences in the relative expression of NPC1 protein without the onset of NPC1 disease, previous studies have demonstrated that NPC1 heterozygous ($NPC1^{+/-}$) mice, which express only intermediate amounts of NPC1 protein in the liver and other tissues, including isolated fibroblasts, do not accumulate cholesterol in these tissues or display any symptoms of NPC1 disease (15, 17, 28). Together, these results suggest that a certain threshold amount of NPC1 protein may be necessary to maintain intracellular cholesterol homeostasis.

Using Northern blot and immunoblot analysis, both NPC1 mRNA and NPC1 protein were detected in every tissue of the mouse, consistent with the observation that cholesterol accumulates in every tissue, including the central

nervous system, of the NPC1 mouse (15, 16, 18). The relative expression of NPC1 mRNA and NPC1 protein measured in a particular tissue was approximately the same, suggesting that the NPC1 gene is regulated primarily at the level of transcription. Consistent with this result, studies have reported a NPC1 promoter region residing between −37 and −111 bp necessary to control promoter activity and the expression of NPC1 (7). In addition, other promoter regions have since been identified residing between −120 and −430 bp that constitute two consensus sequences for the cAMP response element binding protein, in which the constitutive transcription of NPC1 mRNA was enhanced severalfold in response to cAMP through the protein kinase A pathway (57, 58). In light of these results, it is again interesting that only intermediate amounts of NPC1 protein have been detected in the livers and isolated fibroblasts derived from NPC1 heterozygous mice, suggesting that posttranscriptional regulation of NPC1 is not simply a physiological response to the decreased amounts of NPC1 protein (28).

To further investigate the regulation of NPC1 expression in mouse tissues, the relative expression of NPC1 protein in relation to the combined rate of CMr-TC and LDL-TC uptake and the combined rate of HDL-CE uptake and cholesterol synthesis in different tissues of the mouse was determined. As shown in Fig. 1, there are two major pathways for the flow of cholesterol through different cells in the body. One pathway, referred to as the coated-pit pathway, facilitates the uptake of CMr-TC and LDL-TC into coated pits after interaction with the LDLR present on the cell surface (59). Subsequent endocytosis of the lipoprotein receptor complex and hydrolysis of lipoprotein cholesteryl ester within late endosomes/lysosomes by an acidic cholesteryl ester hydrolase results in the release of cholesterol. Furthermore, it has now been determined that enrichment of late endosomes/lysosomes with lipoprotein-derived cholesterol stimulates the interaction of the NPC1 protein with these compartments, supposedly to facilitate the transport of cholesterol to other cellular compartments and ultimately the metabolically active pool of cholesterol (27, 28).

In the present study, the relative expression of NPC1 mRNA and NPC1 protein was determined in relation to the flow of cholesterol through the coated-pit pathway using two different methods. First, the relative expression of NPC1 protein in different tissues of the mouse was compared with the relative uptake of CMr-TC and LDL-TC in these tissues, and the results indicated no correlation. Second, the relative expression of NPC1 mRNA and NPC1 protein in the liver and cerebrum of the mouse was compared with the flow of cholesterol through the coated-pit pathway by supplementing diets with increasing amounts of cholesterol or cholestyramine. As noted in this and previous studies, when mice are fed a diet containing an increased amount of cholesterol, the total concentration of cholesterol in the liver and fecal bile excretion increases, whereas mice fed a diet containing cholestyramine show an increase in cholesterol 7α-hydroxylase activity and fecal bile excretion; together, these results indicate that both increased cholesterol and cholestyramine result in an increased flow of cholesterol through cells in the liver (19, 49). This being true, results from the present study indicate that when mice are fed a diet with increasing amounts of cholesterol or cholestyramine, the relative expression of NPC1 mRNA in the liver is not altered significantly. This result was confirmed by the relative expression of NPC1 protein, in both the liver and cerebrum, not being altered significantly when mice were fed a diet with increasing amounts of cholesterol or cholestyramine. Therefore, this study strongly suggests that the relative expression of NPC1 is not regulated by the flow of cholesterol through the cell and that NPC1 is constitutively expressed in relation to the amount of cholesterol.

In the second pathway that was described in Fig. 1, referred to as the SR-BI pathway, SR-BI facilitates the selective uptake of cholesteryl ester from HDL-CE into the cell. The internalized cholesteryl ester is then hydrolyzed in an undefined compartment, resulting in the release of cholesterol (40, 60). This cholesterol mixes with newly synthesized cholesterol and enters the metabolically active pool of cholesterol destined for excretion from the cell. As studies have already shown, the mutation of NPC1 does not adversely affect the flow of cholesterol through the SR-BI pathway (19). Moreover, as the present study indicates, the relative expression of NPC1 protein is not affected by the rate of cholesterol synthesis or the rate of cholesterol flowing through the SR-BI pathway.

A number of key proteins involved in regulating cholesterol and lipoprotein metabolism, such as the LDLR, HMG-CoA reductase, the ATP binding cassette family of transporters, caveolin, and even the NPC1L1 protein, which shares 42% identity with the NPC1 protein, have been shown to be regulated by sterol through the sterol-regulatory element binding protein and/or peroxisome proliferator-activated receptor and liver X receptor/retinoid X receptor pathways (61–66). However, it is important to emphasize that several other key proteins involved in regulating aspects of cholesterol metabolism, including acidic cholesteryl ester hydrolase, the cholesterol binding protein MLN64 associated with the NPC1 compartment, and ACAT, which is responsible for the esterification of excess cellular cholesterol, are not regulated by the amount of cholesterol, similar to the results presented in this study investigating the expression of NPC1 (30, 67–69).

In conclusion, although the present study has demonstrated that expression of NPC1 is not regulated by the flow of cholesterol through the cell, the NPC1 protein does contain a sterol-sensing domain that is homologous to that encoded within HMG-CoA reductase, SCAP, and NPC1L1 (3, 6, 8). The topology of the NPC1 sterol-sensing domain is similar to that of these other proteins, and it has been determined that the NPC1 sterol-sensing domain must be functional to facilitate cholesterol transport from late endosomes/lysosomes to other cellular compartments (5, 6, 70). Interestingly, it was shown recently that these three proteins (NPC1, HMG-CoA reductase, and SCAP) that contain a sterol-sensing domain are capable of directly binding cholesterol (31, 71, 72). With respect to NPC1,

the presence of cholesterol within late endosomes/lysosomes has an affect upon the cellular location and function of NPC1, presumably through the direct interaction of cholesterol with the sterol-sensing domain (27, 28, 32, 34). Obviously, additional research is needed to delineate how cholesterol affects the function of NPC1 and exactly how NPC1, along with other proteins, regulates the flow of cholesterol through the cell.

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