Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice

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Abstract Lipoprotein metabolism is an important contributing factor in the development and progression of atherosclerosis. Plasma lipoproteins and their receptors are heavily glycosylated and sialylated, and levels of sialic acids modulate their biological functions. Sialylation is controlled by the activities of sialyltransferases and sialidases. To address the impact of sialidase (neu1) activity on lipoprotein metabolism, we have generated a mouse model with a hypomorphic neu allele (B6.SM) that displays reduced sialidase expression and sialidase activity. The objectives of this study are to determine the impact of sialidase on the rate of hepatic lipoprotein secretion and lipoprotein uptake. Our results indicate that hepatic levels of cholesterol and triglycerides are significantly higher in B6.SM mice compared with C57Bl/6 mice; however, VLDL-triglyceride production rate is lower. In addition, B6.SM mice show significantly lower levels of hepatic microsomal triglyceride transfer protein (MTP) and active sterol-regulatory element binding protein (SREBP)-2 but higher levels of diglyceride acyltransferase (DGAT)2; these are all indicative of increased hepatic lipid storage. Rescue of sialidase activity in hypomorphic sialidase mice using helper-dependent adenovirus resulted in increased VLDL production and an increase in MTP levels. Furthermore, hypomorphic sialidase expression results in stabilization of hepatic LDL receptor (LDLR) protein expression, which enhances LDL uptake.

Supplementary key words neu1 • very low density lipoprotein • cholesterol metabolism • adenovirus

Atherosclerosis is a chronic inflammation of the arteries caused by subendothelial accumulation of modified lipoproteins and their interactions with components of the vasculature and immune cells (1). This complex and multifactorial disease is the leading preventable cause of death in the modern world due to complications involved with plaque formation and rupture, which lead to coronary heart disease, myocardial infarctions, and strokes (2). Neu1 sialidase belongs to a family of hydrolytic enzymes that cleave terminal sialyl linkages of glycoproteins, glycolipids, and oligosaccharides (3). While the role of the enzyme in the lysosome is well defined, its functional consequences on the cell surface, including its role in cell-surface receptor recognition, catabolism, tumorigenicity, and antigen masking, are still being explored (4–8). Some of its documented roles include regulation of the function of cell-surface molecules, such as TLR4, CD15, CD22, CD43, CD44, and CD45 (9–17). In inflammatory response, sialidase is involved in modulating the function of macrophages (18–20), T cells (21–23), and neutrophils (24–26), indicating its potential effect in chronic inflammation, such as atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease. To date, genes encoding lysosomal/membrane (neu1) (27–29), cytosolic (neu2) (30, 31), plasma membrane-bound (neu3) (32, 33) and mitochondrial (neu4) (34–36) sialidase have been cloned. A deficiency in lysosomal/membrane sialidase (neu1) is associated with sialidosis, an autosomal recessive disease, which is characterized by accumulation of sialylconjugates within the lysosomes in the central nervous system as well as in peripheral tissues (37). Furthermore, early reports have indicated...
that sialidase can affect LDL metabolism in vitro and that serum sialylation levels can be used as an indicator of cardiovascular disease risk (38–42). Additionally, low-density lipoprotein receptor (LDLR) and apolipoprotein (apo)B, CII, CIII, and E are all heavily sialylated, and several studies postulate that sialic acids can have functional significance on these proteins (43–52). Despite their apparent roles in lipid metabolism and atherosclerotic disease progression, the functional effects of sialic acids on these glycoconjugates have not been significantly addressed. To assess the effects of sialidase on lipoprotein and cholesterol metabolism in vivo, we sought to generate and analyze mice with hypomorphic sialidase expression. A partial deficiency of sialidase was identified in the SM/J mouse in the 1970s (53, 54), and these animals have abnormal sialylation of glycoproteins and an impaired immune response (55). Campbell and colleagues have demonstrated that the SM/J liver expresses low sialidase mRNA (28), and we have recently identified a point mutation (-519G>A) in the mouse lysosomal sialidase promoter (neu1), which results in reduced sialidase gene transcription in the SM/J mice. This promoter mutation creates a binding site for a transcriptional repressor, Nkx3.2, resulting in reduced gene expression (56). SM/J mice, however, harbor mutations in a number of other genes (57, 58), complicating analysis of the physiological consequences of low sialidase. We have therefore isolated this mutation from the SM/J mice by backcrossing onto a C57Bl/6 genetic background, generating a hypomorphic sialidase mouse, named B6.SM, which has reduced sialidase protein levels and activity. In this study, we focus on the effect of hypomorphic sialidase (neu1) expression on the regulation of lipoprotein metabolism in the liver. We demonstrate that hypomorphic sialidase expression lowers serum cholesterol levels by modulating hepatic VLDL production as well as hepatic lipid metabolism. This study points to sialidase as an important player in lipoprotein metabolism and as a potential therapeutic target for metabolic syndrome diseases, such as hypercholesterolemia and atherosclerosis.

METHODS

Mice

B6.SM mice were obtained by crossing SM/J mice with C57Bl/6 mice six times. The presence of the regulatory mutation, (-519G→A) within the neu1 promoter was confirmed by PCR using DNA extracted from tail biopsies. The following primers were used for the PCR: 5′ ATC CTC GTC CAG GAA CTG GT 3′ and 5′ CTT AAG GGC ATT GGG GTC AT 3′. PCR products were digested with MspAI (New England Biolab), which serves as a genetic diagnostic as it only cleaves the PCR product carrying the B6.SM mutation. Mice were housed in microisolator cages in a room with a 12 h light and dark cycle and given unlimited access to food and water. All experimental protocols were approved by our Institutional Animal Research Ethics Committee.

Cell lines

Three human fibroblast cell lines were used: normal (MCH64) and sialidosis (WG544 and W) lines. The cell line W was isolated from a sialidosis patient; it has a premature stop codon (69G>A) in the neu1 gene and null sialidase activity (59). MCH64 and WG544 were obtained from the Montreal Children’s Hospital Research Institute. Cells were cultured in DMEM with 10% FBS and maintained in a 37°C 5% CO2 incubator. OptiMEM Low Serum (Invitrogen) media was used prior to receptor and lipoprotein uptake studies to starve cells and upregulate LDLR. The human embryonic kidney cell line 293 and 293Cre4 were generous gifts from Dr. Frank Graham (McMaster University, Hamilton, ON) and were grown in F11 medium with 10% FBS and maintained in a 37°C 5% CO2 incubator. 293Cre4 cells were grown similarly but with 0.4 mg/mL G418 antibiotic.

Adenoviral infections

All adenovirus was propagated in F11 medium supplemented with 5% horse serum with antibiotics and fungizone. Adenoviral infections were carried out by adding the adenovirus in PBS++ on 90% confluent dishes immediately after removal of cell medium. Adenovirus was allowed to adsorb to the cell monolayer for 1 h.

Cloning of mouse sialidase gene into helper-dependent vector

A genomic fragment containing the mouse lysosomal sialidase gene and promoter was isolated from a BAC (NCBI accession number AF109906) containing 180 kb spanning the sialidase locus (Genome Systems) and was subcloned into a pBSK plasmid (60). pBSKS+msialR plasmid containing the mouse neu1 gene was digested with NotI and RsrII, yielding a 10.6 kb fragment. The helper-dependent plasmid pCH-SU was similarly digested with NotI and RsrII, producing a 19.3 kb fragment containing the adenoviral components necessary for viral encapsulation, the left and right inverted terminal repeats (ITR) and the packaging signal (Ψ). The 10.6 kb mouse sialidase fragment and 19.3 kb adenoviral vector fragment were both purified with the GeneClean II with Spin kit (Q-BIOgene). The 10.6 kb mouse sialidase fragment was then ligated by compatible cohesive ends to the 19.3 kb adenoviral vector fragment, producing a 29.9 kb helper-dependent vector containing the mouse sialidase gene, pCHSUmsial, verified by restriction digestion (Fig. 4A).

Propagation of HD-Ad containing mouse sialidase gene

The helper-dependent plasmid pCHSUmsial was digested with PmeI to release the bacterial amplification elements, ampicillin resistance, and the origin of replication, producing a 27.2 kb fragment and exposing the two ITRs on the ends of the fragment. Following heat inactivation, the digested plasmid was transfected into 293Cre4 cells by calcium phosphate transfection. After adsorption and addition of maintenance medium, complete cytopathic effect (CPE) was seen 48 h postinfection. The cells were then scraped and stored. This lysate was used to coinfect a 90% confluent dish of 293Cre4 cells along with the helper virus at an MOI of 1 PFU/cell. After complete CPE was seen, the cell lysate was harvested and stored. Coinfections continued serially up until eight lysates had been harvested. After each lysate had been collected, pronase/SDS was added to the dishes to digest the remaining adenovirus to confirm correct adenoviral propagation. Phenol extraction and ethanol precipitation purified the adenoviral DNA, and restriction digestion confirmed proper adenoviral sequence. Sequencing of the purified adenovirus DNA for the mouse sialidase promoter also confirmed the presence of the mouse sialidase gene within the viral genome. The helper-dependent vector Ad5C4HSULacZ was provided by...
Chang-Xin Shi (McMaster University, Hamilton, ON) and was serially passaged as above.

**Purification and concentration determination of adenovirus**

After CPE was reached postinfection, cells were scraped into 10 mM Tris-HCl, pH 8.0. Sodium deoxycholate (5%) was added to lyse the cells, followed by the addition of 2M MgCl₂ and DNase I to digest any unpackaged viral DNA as well as cellular DNA. The lysate was then spun, and the supernatant was collected and ultracentrifuged twice through CsCl density gradient (61). The lower viral bands were collected with an 18 gauge needle and syringe through the side of the tube. The collected adenovirus was then injected into a Slide-A-Lyzer dialysis cassettes (Pierce) where the virus was dialyzed against three changes of 500 ml 10 mM Tris-HCl (pH 8.0) over 24 h. The adenovirus was collected from the dialysis cassettes, and sterile glycerol was added to a final concentration of 10%. The concentration of helper-dependent adenovirus was determined through fluorometric analysis using Hoechst dye (Boehringer Mannheim). CsCl-banded adenovirus (20 μl) was treated with 20 μl of pronase/SDS overnight at 37°C to degrade the viral capsid. The following day, 20 μl of the pronase/SDS-treated virus was exposed to the Hoechst dye, and fluorometric analysis was measured using the Hoefer Fluorometer (Hoefer). Adenoviral particle count was based on the fluorometric result (μg/ml) of inserting this value into the following: Viral DNA Concentration (μg/ml) / 9.48 × 10⁻¹¹ / Length of Viral DNA (Kb).

**Adenoviral administration in vivo**

Helper-dependent adenoviruses containing mouse sialidase gene or lacZ cDNA (100 μl; 10⁶ particles/mouse in sterile PBS) were injected into the tail vein of 5-month-old male B6.S.c mice under isoflurane anesthetic. Mice were monitored for the incubation period of 14 days until VLDL-production experiments.

**Collection of blood and tissues**

Mice were anesthetized with ketamine/xylazine and euthanized by exposure of their thoracic cavity. Blood was obtained for 5 min at 15,000 rpm using serum collection tubes (Sarstedt). Serum was obtained by centrifugation of blood by cardiac puncture. Blood was obtained by exposure of their thoracic cavity. Blood was obtained by cardiac puncture. Serum was obtained by centrifugation of blood.

**Sialidase activity assay**

Approximately 0.15 g of tissue was minced on ice and homogenized in 1.5 ml water. Tissue homogenate (50 μl) was then incubated for 1 h at 37°C with 60 μl of 0.4 mM 4-Mu-NANA in acetate buffer (pH 4.2) with 10% BSA. Assay was performed similarly for WG544 cell lysates infected with the HD-Ad sialidase. Activity is measured as the amount of fluorescence generated from the liberation of umbelliferone (4-Mu) from the NANA substrate. The reaction was stopped by the addition of 2 ml of basic 0.1 M MAP buffer. Fluorosence was then measured using a plate fluorometer (PerkinElmer) and normalized to protein concentration.

**RNA isolation and quantitative real-time PCR**

Livers were homogenized in RNA lysis buffer, and then RNA was isolated using Norgen Total RNA Isolation Kit. Total RNA (1–5 μg) was then reverse transcribed using oligoDT primers following the protocol of Invitrogen’s SSIII RT reverse transcriptase. cDNA was then used for qRT-PCR using Applied Biosystems Power Sybr Green. Plates were loaded with a 20 μl reaction per well and included appropriate blanks and standard. (PCR cycle was as follows: 10 min 95°C, 40× 15 sec 95°C, 60 sec 60°C). Primers (synthesized by MOBIX facility, McMaster University) for LDLR qRT-PCR were: 5′TGACTCGACGCAACAGGCTG3′ and 5′ATCTAGGGCATCTCGGTCTCC′ and for SREBP2 qRT-PCR were: 5′GCGCAACAGGGGACCTTCT3′ and 5′CCCCATGACATTGCTTTCAACT3′.

**Immunoblot analysis**

Livers or cells were homogenized in RIPA buffer containing protease inhibitors (Roche), and the protein concentration was determined using the Bradford assay (Bio-Rad). Samples were separated on SDS-polyacrylamide gel and transferred to nitrocellular membranes using Tris-Glycine buffers. The following antibodies were used: anti-MTP (mouse anti-MTP, 1:2500, BD Biosciences); anti-ACAT2 (mouse anti-ACAT2, 1:200, Santa Cruz Biotechnology); anti-SREBP-2 (mouse anti-SREBP-2, 1:500, Pharmigen); anti-SREBP-1a/c (rabbit anti-SREBP-1, 1:1000, Novus); anti-LDLR (mouse anti-LDLR, 1:1000, Calbiochem, and goat anti-LDLR 1:1000, R and D); anti-LRP1 (rabbit anti-LRP1, 1:10000, Epitomics); anti-neul sialidase (rabbit anti-neul 1:500, Rockland); anti-DGAT2 (rabbit anti-DGAT2 1:1000, Novus); anti-GPAPD (goat anti-GPAPD 1:2000, R and D); anti-β-tubulin (mouse anti-β-tubulin, 1:1000, Chemicon International); and anti-β-actin (mouse anti-β-actin, 1:1000, Cell Signaling Technology). Secondary HRP-conjugated antibodies (1:10000, Santa Cruz Biotechnology) were used and visualized with chemiluminescence (ECL, Amersham). Intensity was measured by ImageJ densitometry software.

**Immunoprecipitation and lectin pull-downs**

Liver membrane-enriched lysates with equal amounts of protein were immunoprecipitated using LDLR antibody and Protein A prior to immunoblot analysis, as described before (62). We utilized SNA (Sambucus nigra agglutinin), which binds α 2,6 linkages of sialic acid and MALII (Maackia amurensis leucoagglutinin), which binds α 2,3 linkages of sialic acid (63). The bovine-labeled lectins (Vector Laboratories) were incubated with membrane-enriched lysates and pulled down with streptavidin beads. The enriched samples, containing glycoproteins pulled down by their sialic acids, were used for immunoblot analysis.

**TrueBlot immunoprecipitation of PCSK9**

Serum was incubated with anti mouse PCSK9 antibody (courtesy of Dr. Nabil Seidah, University of Montreal, Montreal, Canada) and TrueBlot agarose anti-rabbit beads (eBioscience) overnight at 4°C. Samples were spun and washed with RIPA buffer with protease inhibitors (EDTA-free). The resulting pellet was boiled in Laemmli Sample Buffer, spun down, and then 30 μl of the supernatant was subjected to SDS-PAGE and blotted with the same antibody. A special secondary antibody (TrueBlot anti-Rabbit IgG HRP, eBioscience), which only detects full-length immunoglobulin, was used on the Western blot to avoid nonspecific bands.

**Lipid analyses**

For hepatic lipid analyses, 150 mg of liver was homogenized in 1 ml of TNE [10 mM Tris (pH 7.5), 400 mM NaCl, 100 mM EDTA, 0.6% SDS]. Folch mixture (chloroform/methanol, 2:1; 3 ml) was added to 300 μl of liver homogenates, and the tubes were mixed for 1 min. After that, 0.6 ml of distilled water was added to the tubes, and the tubes were mixed for 1 min. The extraction mixture was left at 4°C for 2 h. After 2 h, the tubes were centrifuged at low speed to facilitate phase separation. The lower phase (chloroform phase) was dried completely by sitting in a water bath at 37°C. The dry chloroform phase was resuspended in 60 μl of isopropanol. Hepatic total cholesterol was analyzed with enzymatic
assay (Infinity Cholesterol Liquid Stable Reagent, Thermo Scientific). The enzymatic colorimetric assay product was measured at 500 nm. Free cholesterol was analyzed with Free Cholesterol E Reagent (Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm. Cholesterol ester calculation was calculated by subtracting free cholesterol measurements from total cholesterol concentration. Triglyceride was analyzed with enzymatic colorimetric assay (L-Type Triglyceride H, Wako Diagnostics). The absorbance of the reaction product was measured at 600 nm. Serum samples were measured directly as above. For lipoprotein cholesterol analyses, 300 μl of serum was fractionated by gel filtration-FPLC using a Superose 6 column (64), and lipid levels were measured with enzymatic assay as above.

In vivo hepatic VLDL-lipid secretion

Hepatic production of VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl esters were measured in 3-month-old male C57Bl/6 and B6.SM after intravenous injection of Triton WR 1339 (Tyoxapol T0307-10G, Sigma BioXtra, Sigma-Aldrich) (15 g/dl in 0.9% NaCl). Mice were fasted overnight prior to the experiments, and 500 mg/kg mg of Triton WR 1339 was injected. Blood samples were taken from the cheek under light anesthesia before and at 1, 2, 3, and 4 h after Triton injection for triglyceride, cholesterol, free cholesterol, and cholesteryl ester measurements. VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl ester production rates were obtained by calculating the slope of the regression line of the graph with VLDL-triglyceride, cholesterol, free cholesterol, and cholesteryl ester concentrations, respectively, versus time in hours.

Fibroblast Oil Red O staining

For staining of neutral lipids in cells, Oil Red O powder from Sigma-Aldrich (O0625) was prepared by dissolving 2.5 g in 500 ml of isopropanol. Prior to experimental use, this mixture was diluted 3:2 with isopropanol and filtered to remove any particulate matter. Cells were grown on uncoated glass coverslips in a 24-well plate and incubated with 50 μg/ml LDL (BTI Inc.) for 24 h after growing in Optimem for 72 h. Cells were washed, fixed with 3.7% formaldehyde, and then washed with 60% isopropanol prior to Oil Red O staining for 1 h. This was followed by another 60% isopropanol rinse and four PBS washes. Hematoxylin staining was then performed, and the coverslips were mounted on microscope slides using Aqua Mount from Fisher. Pictures were taken using brightfield and phase microscopy with Zeiss Axiovision software. Quantification was performed by measuring the red stained area and dividing by nuclear area per cell and averaging each group.

Immunofluorescence

Cells were grown in Optimem for 72 h then fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton-X 100 in PBS. Cells were blocked in 20% goat serum in PBS, incubated overnight with anti-LDLR antibody (R and D) at 1:400, washed, incubated with Texas Red anti-goat secondary at 1:400, stained with DAPI, and then mounted using ProLong Gold Antifade reagent from Invitrogen. Images were taken and analyzed with AxioVision software from Zeiss.

Fibroblast cholesterol assay

Fibroblasts were grown in Optimem for 72 h then incubated with 50 μg/ml LDL for 24 h. Briefly, cells were washed and lipids were extracted using a hexane/isopropanol mixture overlaid on the cells for 1 h. The mixture was allowed to evaporate and lipids were resuspended in 100 μl isopropanol for enzymatic measurements of cholesterol as described above. After lipid extraction, cells were scraped in 0.1% SDS, and protein content was measured using a Lowry assay to normalize cholesterol readings.

Statistical analysis

Statistical analyses between multiple groups of data were analyzed by one-way ANOVA followed by Tukey comparison test and multiple comparison test using Prism 5 (version 5.04). Statistical analyses between two groups were performed using an unpaired Student’s t-test. Error bars represent SEM unless otherwise noted. Data were considered statistically different if P < 0.05.

RESULTS

Sialidase protein expression and activity in B6.SM mice

Western blot analysis of hepatic neu1 sialidase reveals a significant reduction (approximately 85%) in sialidase expression in B6.SM compared with C57Bl/6 mice (Fig. 1A). Sialidase activity is significantly reduced in the brain, liver, spleen, and kidney of B6.SM mice (Fig. 1B). The sialidase enzymatic assay of organ

![Fig. 1. Sialidase protein expression and activity in C57Bl/6 and B6.SM mice. (A) B6.SM males show a significant reduction in hepatic neu1 sialidase expression compared with C57Bl/6 controls (P = 0.03). Representative blots of n = 3 for each group. Liver lysates were subjected to SDS-PAGE (8%). Membranes were probed with anti-neu1 Sialidase antibody and anti-β-actin as a control. Intensities of bands were measured by ImageJ densitometry software. (B) B6.SM tissues have significantly lower levels of sialidase activity, and this is especially prominent in the liver where levels are reduced to approximately 20% of C57Bl/6. Brain, liver, spleen, and kidney lysates were assessed for sialidase activity using fluorescent 4-Mu-NANA. *P = 0.01, **P = 0.001, ***P < 0.0001. mU = μMol/hr.](https://example.com/fig1.png)
extracts from B6.SM mice indicated that this strain recapitulates a tissue-specific hypomorphic sialidase mouse model.

**Effect on serum cholesterol levels and hepatic lipid levels**

Measurement of fasted serum levels of total cholesterol and triglycerides revealed no significant difference between C57Bl/6 and B6.SM mice (Table 1). However, when serum lipoproteins from unfasted male mice were fractionated by size exclusion using fast-protein liquid chromatography (FPLC) with a superose 6 gel filtration column (Fig. 2), the cholesterol profiles of B6.SM mice show significantly lower cholesterol levels in LDL fractions (fractions 20–30) than those of the corresponding C57Bl/6 controls. Also, the HDL peak shows a slight shift indicative of smaller-sized HDL particles in B6.SM mice (64–66) (Fig. 2). Additionally, the VLDL peak appears to be slightly smaller in B6.SM mice (Fig. 2) and to shift to the right, indicative of smaller-sized VLDL particles (Fig. 2, inset). These results indicate that hypomorphic sialidase expression appears to lower cholesterol levels of LDL-sized particles. To determine whether the altered cholesterol profile is associated with altered hepatic lipid metabolism, we measured the hepatic total cholesterol, triglyceride, free cholesterol, and cholesteryl ester content in both B6.SM and C57Bl/6 mice. We observed a significant increase in hepatic total cholesterol, cholesteryl esters, and triglyceride in B6.SM mice compared with C57Bl/6 mice (Table 2). A trend of increase in hepatic free cholesterol of B6.SM mice compared with C57Bl/6 mice is also noted. These results point to sialidase activity as having a role in modulation of lipid metabolism and homeostasis in the liver.

**In vivo effect on hepatic VLDL-lipid production and MTP expression**

To determine whether the changes in serum and hepatic cholesterol levels are caused by a decrease in VLDL-lipid production, we have measured the lipid concentrations at several intervals post lipoprotein lipase inhibition. Administration of Triton WR1339 prevents the hydrolysis of lipid production, we have measured the lipid concentrations at several intervals post lipoprotein lipase inhibition. Administration of Triton WR1339 prevents the hydrolysis of lipoprotein metabolism initiated by reductions in MTP expression. Because the decrease in VLDL-triglyceride appears to be a direct result of the decreased protein expression of MTP, these data show that B6.SM mice exhibit a drastically altered lipoprotein metabolism initiated by reductions in VLDL assembly and production.

**Hepatic VLDL production and MTP expression after sialidase gene therapy in B6.SM mice**

The mouse lysosomal sialidase gene (10.6 kb) was ligated via compatible cohesive ends into the 19.3 kb adenoviral vector pC4HSU that contained the two ITRs and the packaging signal (VP). The resultant plasmid, pC4HSU-msial, was confirmed by digesting with EcoRI, yielding the expected fragment sizes (Fig. 4A). After the helper-dependent mouse sialidase adenovirus was properly characterized and CsCl purified (see Methods), we analyzed its effects on sialidase activity. Sialidosis fibroblast cells were infected with increasing doses of viral particles, which caused a steady, almost linear increase in sialidase activity with increasing virus concentration (Fig. 4B). These results demonstrate that this helper-dependent mouse sialidase adenovirus (HD-AdSial) adenovirus is a functional vector capable of producing active mouse neu1 sialidase in vitro. Because B6.SM male mice have lower VLDL-TG production compared with C57Bl/6 controls (Fig. 3), we sought to determine whether adenoviral sialidase gene therapy would rescue the phenotype in B6.SM animals. Thus, we infected B6.SM sialidase-deficient male mice with helper-dependent mouse sialidase (HD-AdSial) or LacZ (HD-AdlacZ) adenovirus, and then measured VLDL-TG production. The HD-AdSial group had significantly higher expression of neu1 sialidase protein in the liver compared with the HD-AdlacZ group, confirming expression of the virus (Fig. 4C). B6.SM mice infected with sialidase virus had significantly higher VLDL-TG production compared with LacZ controls (Fig. 4D). These data directly demonstrate that

**Table 1. Serum lipid levels in C57Bl/6 and B6SM mice**

|            | Serum Total Cholesterol (mM) | Serum Triglycerides (mM) |
|------------|------------------------------|-------------------------|
| C57Bl/6 Male | 3.33 ± 0.94                  | 0.63 ± 0.25              |
| B6.SM Male  | 2.97 ± 0.29                  | 0.52 ± 0.10              |

Fasted serum levels of cholesterol and triglycerides from six-week-old C57Bl/6 and B6SM males on a standard chow diet (n = 3). Mean ± SD are shown.
rescuing sialidase deficiency via adenovirus increases VLDL-TG production, similar to what was observed in C57Bl/6 mice. The HD-AdSial group also had an increase in hepatic MTP protein (Fig. 4E) compared with the LacZ controls, indicating that sialidase can affect MTP levels, although the mechanism is yet to be determined. These findings enable us to conclude that low levels of neu1 sialidase in B6.SM mice are directly driving reduced VLDL-TG production.

**Hypomorphic sialidase expression decreases hepatic SREBP-2 and increases hepatic DGAT2 expression**

To investigate the mechanisms behind the changes in hepatic lipid levels and lipoprotein metabolism, we analyzed protein expression of several important enzymes and transcription factors. We found a significant decrease in cleaved hepatic sterol-regulatory element binding protein (SREBP)-2 expression in B6.SM mice (Fig. 5A). It has been shown that the MTP promoter contains SREBP-2 response elements (70, 71), suggesting that reduced SREBP-2 may contribute to the reduced MTP levels. Nevertheless, higher levels of cholesterol in the livers of B6.SM mice reduce active SREBP-2 and affect downstream gene expression. Additionally, protein levels of cleaved SREBP-1α/β remain unchanged between the two strains (Fig. 5B). It appears that the phenotype observed in B6.SM livers is primarily due to SREBP-2 and independent of SREBP-1. Furthermore, we assessed the expression of hepatic acylCoA:cholesterol acyltransferase (ACAT-2), which mediates esterification of hepatic cholesterol (72), and we found a trend of higher ACAT-2 expression, implying that there is sufficient excess of cholesterol to be esterified (Fig. 5C). Due to the increases in triglyceride levels observed in the livers of B6.SM mice, it was important to analyze the diglyceride acyltransferase

![Fig. 3. In vivo hepatic VLDL-TG production in C57Bl/6 and B6.SM mice. C57Bl/6 (n = 3) and B6.SM mice (n = 3) were fasted overnight and injected with the lipoprotein lipase inhibitor Triton WR1339 (500 mg/kg). Serum samples were drawn just before the injection (time 0 h) and at 1, 2, 3, and 4 h postinjection. There is a decrease in serum (A) VLDL-triglyceride, (B) VLDL-cholesterol, (C) VLDL-free cholesterol, and (D) VLDL-cholesterol esters concentrations at different indicated time points after 0 h (*P < 0.05). Also note that there is a decrease in the steepness of the slope of the B6.SM mice compared with that of the C57Bl/6 mice. This indicates that hypomorphic sialidase expression causes decreased hepatic VLDL-TG production rates. Values represent means ± SE. (E) B6.SM mice have a significant decrease in the protein expression of MTP compared with C57Bl/6 (P = 0.002), which further supports that hypomorphic sialidase expression results in decreased VLDL-TG production. Mouse liver lysates were subjected to SDS-PAGE (8%), and membranes were probed with anti-MTP and anti-β-actin antibodies. Intensities of bands were measured by ImageJ densitometry software.](image-url)
gene that affects the turnover of the LDLR (74–77). Our observation is consistent with the idea that reduced sialidase expression leads to increased LDLR turnover. However, the lower level of LDLR protein expression in B6.SM mice compared with C57Bl/6 mice is likely due to other factors, such as differences in sialic acid expression levels or changes in the interaction of LDLR with other cellular components.

We have measured the serum protein expression of proprotein convertase subtilisin/kexin 9 (PCSK9), a SREBP-2 target gene, in B6.SM and C57Bl/6 mice. The decrease in PCSK9 protein expression observed in B6.SM mice is consistent with a decrease in LDLR transcript, as the LDLR promoter contains SREBP-2 response elements. Thus, the maintenance at the protein level caused by hypomorphic sialidase expression leads to increased LDLR turnover, which in turn could affect the trafficking, degradation, or turnover of the receptor.

**Modulation of hepatic expression of LDLR**

To determine whether the decrease in serum cholesterol levels and the increase in hepatic cholesterol levels in B6.SM mice are caused by altered expression of lipoprotein receptors, we have evaluated the expression of LDLR and LRP-1. Although there is no significant difference in hepatic levels of LDLR or LRP-1 in B6.SM male mice compared with C57Bl/6 controls (Fig. 6A, B), there is a significant reduction in LDLR mRNA levels as measured by qRT-PCR (Fig. 6C). The decrease in SREBP-2 protein expression observed in B6.SM livers is consistent with a decrease in LDLR transcript, as the LDLR promoter contains SREBP-2 response elements. Thus, the maintenance at the protein level caused by hypomorphic sialidase expression could be due to a posttranslational mechanism, such as slower receptor trafficking/recycling or decreased degradation. In view of these results, we have measured the serum protein expression of proprotein convertase subtilisin/kexin 9 (PCSK9), a SREBP-2 target gene that affects the turnover of the LDLR (74–77). Our results indicate that B6.SM mice show a decrease in the serum levels of PCSK9 compared with C57Bl/6 mice (Fig. 6D). PCSK9 gets secreted and can bind to LDL receptors at the cell surface and target them for degradation instead of recycling (78). Therefore, the lower level of PCSK9 expression may result in an increase in the rate of receptor recycling and retention of LDLR protein, despite lower mRNA levels. To determine whether hypomorphic sialidase expression affects the sialylation of LDLR directly, we performed lectin pull-downs followed by Western blotting. Membrane-enriched liver lysates were pulled down with streptavidin beads using biotin-labeled SNA or MAL II, which bind specific α-2,6 and α-2,3 linkages of sialic acid, respectively. This was followed by LDLR blotting to assess how much LDLR was pulled down via the sialic acids. We included control samples immunoprecipitated with LDLR and blotted for total LDLR to ensure equal starting amounts. We observed higher levels of LDLR-associated sialic acids in livers of B6.SM mice compared with C57Bl/6 (Fig. 6E). This indicates that sialic acid molecules on LDLR are directly affected by reduced sialidase expression, with potential functional consequences. Thus, hypomorphic sialidase expression leads to hyperglycosylation of LDLR, which in turn could affect the trafficking, degradation, or turnover of the receptor.

**Sialidase-null mutation increases LDL uptake in human fibroblasts**

To address the direct functional effect of sialidase on LDLR, we utilized human fibroblasts that have null neu1 sialidase activity. The aim of this experiment was to complement the in vivo data with human cells in vitro. We observed no differences in LDLR protein expression between normal and sialidase-null cells, but sialidase-null cells had slightly higher molecular weight LDLR protein, potentially due to hyperglycosylation (Fig. 7A). Furthermore, LDLR immunofluorescence has shown that the receptors appear to cluster next to the nucleus to a greater extent in the sialidosis cell line (Fig. 7B, arrows), although there were no gross changes in expression. To measure the function of the LDLR, we analyzed LDL uptake and lipid droplet formation via Oil Red O staining. Both cell types showed minimal staining during serum starvation but exhibited lipid droplet formation after 24 h of LDL treatment, indicating significant LDL uptake. Neutral lipid accumulation appeared to be slightly higher (although not significant) in sialidase-null versus normal control cells (Fig. 7C), as measured by Oil Red O quantification. To assess this uptake more quantitatively, we utilized lipid extraction followed by enzymatic cholesterol level measurements in these cells. Both cell types had significant increases in cholesterol levels when treated with LDL, indicating internalization of the lipoprotein. However, sialidase-null fibroblasts treated with LDL had significantly higher total cholesterol levels than wild-type cells treated with LDL (Fig. 7D). These data indicate that the absence of neu1 sialidase activity results in higher cholesterol levels due to increased LDL uptake in sialidase-null fibroblasts, consistent with our findings in livers of B6.SM mice.

**DISCUSSION**

In this study, we examined lipoprotein metabolism in a unique mouse model expressing hypomorphic levels of sialidase (neu1). Although sialylation of lipoproteins and lipoprotein receptors has been invoked previously as an important determinant in cholesterol metabolism, little has been reported toward dissecting the impact of sialidase (neu1) on lipoprotein production or clearance in vivo.
phenotype is a direct result of the hypomorphic neu1 gene, we transduced neu1 sialidase expression using helper-dependent adenovirus in B6.SM livers and were able to show a significant increase in both VLDL-TG production and hepatic MTP protein levels compared with HD-AdlacZ controls.

To dissect the mechanisms of increased hepatic lipid storage and decreased VLDL-production in B6.SM mice, hepatic cholesterol and triglyceride metabolism in the liver were investigated. The SREBP transcription factors are master regulators of hepatic lipid homeostasis (79). Our observations of higher hepatic cholesterol levels resulting in lower active SREBP-2 and lower VLDL production phenotype is a direct result of the hypomorphic neu1 gene, we transduced neu1 sialidase expression using helper-dependent adenovirus in B6.SM livers and were able to show a significant increase in both VLDL-TG production and hepatic MTP protein levels compared with HD-AdlacZ controls.

To dissect the mechanisms of increased hepatic lipid storage and decreased VLDL-production in B6.SM mice, hepatic cholesterol and triglyceride metabolism in the liver were investigated. The SREBP transcription factors are master regulators of hepatic lipid homeostasis (79). Our observations of higher hepatic cholesterol levels resulting in lower active SREBP-2 and lower VLDL production...
Sialidase modulates lipoprotein production and uptake. It appears that the phenotype observed in B6.SM livers is primarily due to SREBP-2 and is independent of SREBP-1a/c. In addition, higher levels of hepatic esterified cholesterol as a result of high levels of ACAT-2 expression were observed in hypomorphic sialidase mice. ACAT-2 is an ER-bound enzyme that forms cholesterol esters from cholesterol. ACAT-2 activity decreases the solubility of cholesterol and prevents its incorporation into lipid membranes. As ACAT-2 expression is limited to hepatocytes and enterocytes, ACAT2-derived cholesteryl esters can be packaged directly into VLDL via MTP or stored as neutral lipid droplets in the cytosol. The latter option is more likely in our model as B6.SM mice show lower production levels of VLDL and higher hepatic cholesterol content. We also observed an increase in DGAT2 protein in the livers of B6.SM mice compared with C57Bl/6, which is indicative of increased triglyceride synthesis. These results, along with higher levels of hepatic triglyceride, favor the idea that these animals have increased lipid droplet formation and triglyceride storage, as DGAT does not affect the VLDL production rate. Interestingly, DGAT2 is insensitive to SREBP regulation. Clearly there are other factors that influence levels of DGAT2; nevertheless, changes in DGAT2 and SREBP-2 protein levels contribute to alterations in hepatic lipid homeostasis in hypomorphic sialidase mice. Overall, the decrease in VLDL-lipid production rate observed in B6.SM mice is caused by decreased MTP expression, which appears to be caused by increased hepatic retention of lipids and downregulated SREBP-2 levels.

In addition to VLDL production, hepatic cholesterol content is primarily dependent on LDL endocytosis via LDLR or on chylomicron uptake via LRP-1. The stabilized levels of hepatic LDLR protein in the hypomorphic sialidase mice appear to be due to posttranslational events, as the LDLR mRNA level is in fact lower in B6.SM mouse livers.
more toward differences in ligand-receptor interactions between sialidase-null and normal fibroblasts, as earlier reports had suggested dependency on sialylation for LDL uptake in vitro (38). This also provides verification that sialidase can affect LDLR function directly and complements the mechanism delineated in vivo. These human fibroblast data provide strong evidence for the role of sialidase in LDLR function and suggest a role for sialidase in human lipoprotein metabolism.

Overall, we demonstrate that the changes in lipoprotein metabolism observed in hypomorphic sialidase mice are mediated by a decrease in the production of VLDL-TG in the liver, which is driven by reduced MTP expression and also by hepatic retention of cholesterol and triglycerides. Despite having similar levels of LDLR protein in the liver, hypersialylated LDL receptors in the B6.SM mice are more effective in internalizing LDL, and as a result, hypomorphic sialidase mice show lower serum LDL cholesterol. Although the exact mechanism is unknown, sialylation of LDLR appears to affect...
its recycling or internalization, potentially through PCSK9. Taken together, these events are expected to lead to an athero-
protective effect through the lowering of LDL cholesterol in
the serum. These findings provide evidence of a central role
for sialidase in cholesterol metabolism and set the stage for
examining polymorphisms in the human neu1 gene and
potential links to cardiovascular disease.

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