Research Article

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Transgenic overexpression of Niemann-Pick C2 protein promotes cholesterol gallstone formation in mice

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Background & Aims: Niemann-Pick C2 (NPC2) is a lysosomal protein involved in the egress of low-density lipoprotein-derived cholesterol from lysosomes to other intracellular compartments. NPC2 has been detected in several tissues and is also secreted from the liver into bile. We have previously shown that NPC2-deficient mice fed a lithogenic diet showed reduced biliary cholesterol secretion as well as cholesterol crystal and gallstone formation. This study aimed to investigate the consequences of NPC2 hepatic overexpression on liver cholesterol metabolism, biliary lipid secretion, gallstone formation and the effect of NPC2 on cholesterol crystallization in model bile.

Methods: We generated NPC2 transgenic mice (*Npc2.Tg*) and fed them either chow or lithogenic diets. We studied liver cholesterol metabolism, biliary lipid secretion, bile acid composition and gallstone formation. We performed cholesterol crystallization studies in model bile using a recombinant NPC2 protein.

Results: No differences were observed in biliary cholesterol content or secretion between wild-type and *Npc2.Tg* mice fed the chow or lithogenic diets. Interestingly, *Npc2.Tg* mice showed an increased susceptibility to the lithogenic diet, developing more cholesterol gallstones at early times, but did not show differences in the bile acid hydrophobicity and gallbladder cholesterol saturation indices compared to wild-type mice. Finally, recombinant NPC2 decreased nucleation time in model bile.

Conclusions: These results suggest that NPC2 promotes cholesterol gallstone formation by decreasing the cholesterol nucleation time, indicating a pro-nucleating function of NPC2 in bile.

Keywords: Lithiasis; NPC2; Lithogenic diet; Gallbladder.

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Abbreviations: NPC2, Niemann-Pick C2; NPC1, Niemann-Pick C1; NPC2(h/h), Niemann-Pick C2-deficient hypomorph mice; ABCG5/G8, adenosine triphosphate–binding cassettes G5 and G8; ConA, Concanavalin A; *Npc2.Tg*, Niemann-Pick C2 transgenic mice; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; HEK, human embryonic kidney; BSA, bovine serum albumin; ALT, alanine transaminase; Abcb11, ATP-binding cassette, sub-family B member 11; Abcb4, ATP-binding cassette, sub-family B member 4; CSI, cholesterol saturation index; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; IMAC, immobilized metal affinity chromatography; ELISA, enzyme-linked immunosorbent assay.

Introduction

Niemann-Pick C2 (NPC2) is a lysosomal soluble protein that binds cholesterol with nanomolar affinity and 1:1 stoichiometry through its aliphatic chain [1–3]. *In vitro* assays have shown that NPC2 mediates the transfer of cholesterol between liposomes and that this transfer is accelerated in the presence of anionic phospholipids, such as those present in lysosomes [4,5]. In cells, NPC2 participates with the transmembrane protein Niemann-Pick C1 (NPC1) in cholesterol efflux from low density lipoprotein receptor-mediated endocytosis [6]. In the currently accepted model NPC2 binds cholesterol obtained from the endocytic pathway during and/or after acid lipase hydrolysis and delivers the cholesterol to NPC1, which mediates transfer through the lysosomal membrane to other cell compartments [1,6]. In addition, NPC2 is found in several fluids, including epididymis fluid, milk, plasma and bile [7–9]. In mice epididymis, NPC2 is involved in adding cholesterol to spermatozoa during maturation and its deficiency in spermatozoa reduced their ability to fertilize cumulus-oocytes complexes [10].

In mammals, the liver is a key organ for cholesterol homeostasis. One of the major uptake mechanisms of lipoprotein cholesterol into the liver is the receptor-mediated endocytic pathway. Additionally, hepatocytes eliminate sterols through bile as unesterified cholesterol and bile acids [11]. Biliary cholesterol disposal is critical not only for normal body cholesterol homeostasis but also for the pathogenesis of cholesterol gallstones, a highly prevalent and costly disease condition in Western countries [12]. We have previously reported that NPC2 is expressed in the liver and is secreted into the bile in human and mice. In fact, biliary NPC2 levels were increased in gallstone-susceptible C57BL6/J mice compared to a gallstone-resistant BALB/c strain [9]. In addition, Yamanashi *et al.* found a positive association between NPC2 and cholesterol levels in human bile [13]. Recently, we described

that NPC2-deficient hypomorph mice [NPC2(h/h)] fed a chow diet showed increased biliary cholesterol and phospholipid secretions. In contrast, NPC2(h/h) mice fed a lithogenic diet showed reduced biliary cholesterol secretion and gallbladder bile cholesterol saturation, leading to resistance to cholesterol gallstone formation [14]. This work indicates that NPC2 expression is an important factor in the regulation of diet-derived cholesterol metabolism and in diet-induced cholesterol gallstone formation in mice. Additionally, NPC2 was described as a positive regulator of biliary cholesterol secretion via stimulation of ABCG5/8-mediated cholesterol transport using adenovirus infection in mice [13].

Liver cholesterol hypersecretion into bile is the primary pathogenic event for cholesterol crystallization, but other factors contribute to the development of gallstones, including gallbladder stasis [15,16], which refers to a diminution of gallbladder contraction, and the balance between anti-nucleating and pro-nucleating factors [17–19]. These pro-nucleating factors could explain why bile from patients with the same degree of cholesterol saturation has large differences in cholesterol crystallization speed and the number of gallstones [17,20,21]. Some proteins found in the bile, like IgG, haptoglobin, mucin and aminopeptidase N, promote cholesterol crystallization *in vitro* [22–24]. However, only mucin has been demonstrated to have pro-nucleating activity *in vivo* and patient correlations [25–28]. Interestingly, NPC2 is part of the group of bile glycoproteins that bind to Concanavalin A (ConA) [9], acting as a potent activator of cholesterol crystallization [20,29] and therefore, NPC2 could have a pro-nucleating function in bile. Of note, 59 different proteins were identified by ConA affinity purification, including haptoglobin and mucin-2, in a proteomic analysis of human bile [30]. However, NPC2 was not detected as a ConA binding protein in this study.

The results obtained suggest that NPC2 has a function in the liver modulating lipids secretion, but its role in bile has not been determined. Here, we investigate whether overexpression of the *Npc2* gene in the liver influences hepatic cholesterol metabolism, biliary lipid secretion and diet-induced gallstone formation. We developed a new *Npc2* transgenic strain in a gallstone-susceptible C57BL6/J genetic background. In addition, to analyze the function of biliary NPC2, we studied the effect of NPC2 on cholesterol crystallization in model bile. No differences were observed in biliary cholesterol concentration or secretion between *Npc2.Tg* and wild-type mice fed chow or lithogenic diets. Interestingly, *Npc2.Tg* mice showed an increased susceptibility to the lithogenic diet, developing more cholesterol gallstones at early times, but showed no differences in the gallbladder cholesterol saturation index (CSI) compared to wild-type mice. In addition, recombinant NPC2 decreased the nucleation time in model bile.

Materials and methods

Animals and diet

To generate NPC2 transgenic mice, mouse Npc2 cDNA was cloned into the pLIV7 plasmid, provided by Dr. John M. Taylor (Gladstone Institute Of Cardiovascular Disease, University of California, USA) [31]. The construct was used for zygote microinjection in Centro de Estudios Científicos, Valdivia, Chile.

Transgenic mice were bred with C57BL6/J mice to produce wild-type and NPC2 transgenic mice (*Npc2.Tg*). The offspring were screened for the presence and orientation of the transgene via PCR of genomic DNA from mice tails and Southern blot, as detailed in Supplementary materials and methods.

All mice had free access to water and chow diet (0.02% cholesterol; Prolab RMH 3000, PMI Feeds, St. Louis, MO, USA) until they were used for the studies. For the experiments, 6 week-old *Npc2.Tg* and wild-type male mice were fed chow or lithogenic diets (TestDiet, St. Louis, MO, USA) (15% fat, 1.25% cholesterol and 0.5% cholic acid) for 14, 21 or 28 days. All animals were fasted for 2 h before bile, blood and liver sampling. All protocols were approved by our institution's review board for animal studies and were in agreement with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals recommended by the Institute for Laboratory Animal Research in its Guide for Care and Use of Laboratory Animals.

Hepatic and gallbladder bile and liver sampling

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine at 80–100 and 5–10 mg/kg, respectively. Thereafter mice were euthanized and liver samples and bile were processed as described previously in detail [32].

Hepatic and biliary lipid analyses

Hepatic cholesterol content was analyzed after lipid extraction [32]. Hepatic and gallbladder bile cholesterol and bile acids were measured by enzymatic methods [33,34] and phospholipids were assessed by a colorimetric method [35]. Gallbladder bile CSI was calculated from Carey's critical tables [36], using biliary lipid measurements performed after 14 days of feeding with a lithogenic diet and before mice developed gallstones. Bile acid composition was measured using liquid chromatography-mass spectrometry as described in [37] using hepatic bile obtained after 14 days of feeding with a chow or lithogenic diet. The bile acid hydrophobicity indices were calculated according to Heuman [38].

Gallstone Formation in vivo

After 2 weeks of consuming a lithogenic diet, gallbladder bile was taken and evaluated for the presence of microscopic and macroscopic characteristics of gallstone formation.

Gene and protein expression analyses

Real-time polymerase chain reactions (qPCR), SDS-PAGE, immunoblotting immunofluorescence, quantification of total protein and NPC2 concentration in bile were performed as described in detail in the Supplementary materials and methods section.

Cell transfection and purification of recombinant NPC2 protein

HEK293 cells were transfected transiently with the plasmid containing Npc2 wild-type mouse cDNA donated by Dr. Matthew Scott (Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, USA) [3], or with the plasmid without insert. The conditioned medium was collected and used to purify the NPC2-myc-His protein, as detailed in Supplementary materials and methods.

Preparation of model bile

The model bile was prepared with sodium taurocholate, cholesterol and phosphatidylcholine, all obtained from Sigma-Aldrich (St. Louis, MO, USA). The details of the protocol are in Supplementary materials and methods.

Nucleation assays

Model bile was incubated with wild-type recombinant NPC2 protein, mucin, bovine serum albumin (BSA) and Ni-Affinity proteins obtained from cells transfected with an empty vector.

The time of nucleation and the number of cholesterol crystals were evaluated, as detailed in Supplementary materials and methods.

Immunoprecipitation experiments

The purified recombinant NPC2 protein was immunoprecipitated with anti-NPC2 antibody (Sigma-Aldrich) and protein G PLUS-agarose sc-2002 (Santa Cruz Biotechnology, Dallas, TX, USA). The crystallization-promoting activity of the