



Published in final edited form as:

Steroids. 2020 December ; 164: 108725. doi:10.1016/j.steroids.2020.108725.

Delineation of metabolic responses of *Npc1*^{-/-nih} mice lacking the cholesterol-esterifying enzyme SOAT2 to acute treatment with 2-hydroxypropyl- β -cyclodextrin

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Abstract

Lipids present in lipoproteins cleared from the circulation are processed sequentially by three major proteins within the late endosomal / lysosomal (E/L) compartment of all cells: lysosomal acid lipase (LAL), Niemann-Pick (NPC) C2 and NPC1. When all three of these proteins are functioning normally, unesterified cholesterol (UC) exits the E/L compartment and is used in plasma membrane maintenance and various pathways in the endoplasmic reticulum including esterification by sterol O-acyltransferase 2 (SOAT2) or SOAT1 depending partly on cell type. Mutations in either *NPC2* or *NPC1* result in continual entrapment of UC and glycosphingolipids leading to neurodegeneration, pulmonary dysfunction, splenomegaly and liver damage. To date, the most effective agent for promoting release of entrapped UC in nearly all organs of NPC1-deficient mice and cats is 2-hydroxypropyl- β -cyclodextrin (2HP β CD). The cytotoxic nature of the liberated UC triggers various defenses including suppression of sterol synthesis and increased esterification. The present studies, using the *Npc1*^{-/-nih} mouse model, measured the comparative quantitative importance of these two responses in the liver versus the spleen of *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice in the 24 h following a single acute treatment with 2HP β CD. In the liver but not the spleen of both types of mice suppression of synthesis alone or in combination with increased esterification provided the major defense against the rise in unsequestered cellular UC content. These findings have implications for systemic 2HP β CD treatment in NPC1 patients in view of the purportedly low levels of SOAT2 activity in human liver.

Graphical Abstract

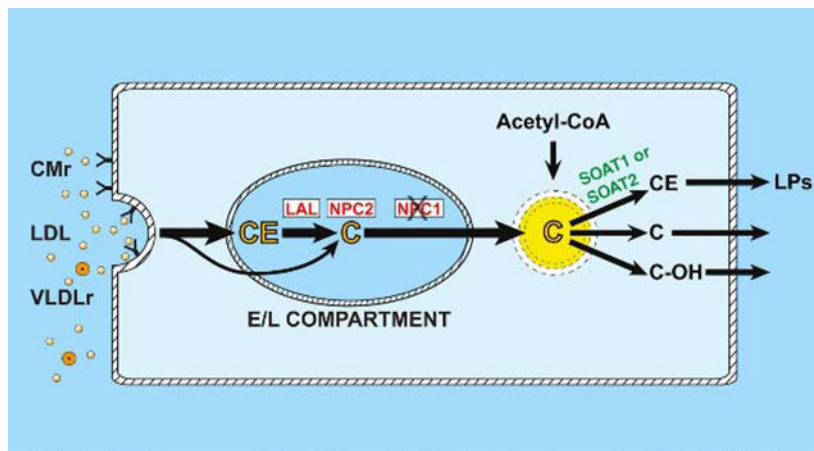
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Conflict of Interest

None of the authors have any conflicts of interest to declare.

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In NPC1 deficiency, unesterified cholesterol (C), derived from lipoproteins (CMr, LDL, VLDLr), becomes trapped in the E/L compartment of cells. 2-Hydroxypropyl- β -cyclodextrin releases sequestered C causing compensatory changes in the rates of cholesterol synthesis (from Acetyl-CoA) and esterification by SOAT1 or 2.

Keywords

Lysosomal storage disease; Unesterified cholesterol sequestration; Cholesterol synthesis and esterification; Liver; Spleen

1. Introduction

Three proteins, lysosomal acid lipase (LAL), Niemann-Pick C (NPC) 2, and NPC1 facilitate the sequential trafficking of lipoprotein-derived cholesterol through the late endosomal/lysosomal (E/L) compartment for subsequent delivery to the endoplasmic reticulum [1,2]. Mutations in either *NPC1* or *NPC2* result in continual E/L entrapment of unesterified cholesterol (UC) and glycosphingolipids [3]. This sequestration of lipids, particularly of UC, can lead to varying degrees of neurodegeneration, pulmonary dysfunction, splenomegaly and liver damage [4]. The number of mutations in *NPC1* far exceeds that in *NPC2* [5]. Globally, the incidence of NPC disease (NPCD) is estimated to be at least 1:120,000 live births [4].

As recently discussed in a comprehensive review [6], a diverse array of treatment strategies for NPC1 deficiency has been evaluated in cell and animal models as well as patients. One particular compound, 2-hydroxypropyl- β -cyclodextrin (2HP β CD), has shown the best prospects for the effective management of NPC1 disease in multiple organs including the central nervous system. Historically, the laboratory of Dr. Robert Erickson was the first to investigate the effects of intraperitoneal 2HP β CD administration on liver cholesterol storage and neurological symptoms in *Npc1*^{-/-} mice [7,8]. However, it was the outcome of later research directed by Dr. John Dietschy showing a single subcutaneous (sc) injection of 2HP β CD in 7-day old *Npc1*^{-/-} mice significantly slowed neurodegeneration and increased lifespan [9] that is now acknowledged as the first description of the use of cyclodextrin as a potential therapeutic agent for NPC1 disease [10]. Other studies at that time by Davidson et

al. [11] demonstrated additional benefit from chronic cyclodextrin administration in *Npc1^{-/-}* mice, thus further propelling research into the potential of this agent for treating NPCD in humans. Over the ensuing years, research in numerous laboratories worldwide defined how 2HP β CD impacted cholesterol metabolism at a cellular, biochemical and molecular level in multiple organ systems of animal models of NPC1 and NPC2 disease [12,13]. Of particular significance was the demonstration that intracerebroventricular administration of 2HP β CD to very young *Npc1^{-/-}* mice [14], and intracisternal delivery of this agent in a feline model of NPC1 disease [15], prevented Purkinje cell death and delayed neurodegeneration. A subsequent non-randomised, open-label, phase 1–2 trial in NPC1 patients demonstrated that intrathecal delivery of 2HP β CD decreased neurological disease progression [16].

The mechanism(s) by which 2HP β CD facilitates release of entrapped UC is not fully understood [13] but in one proposed model it is delivered via pinocytosis to the late E/L compartment where it performs the functions of NPC2 and NPC1 in facilitating the exit of UC [17]. Irrespective of how the release is orchestrated, cells must respond expeditiously to the acute rise in their UC content because of its potential toxicity [18]. While suppression of local cholesterol synthesis provides a rapid and efficient defense mechanism, other adaptations can also come into play. These include esterification [19] and various cellular efflux mechanisms [20]. In tissues there are two cholesterol esterifying enzymes, one of which is sterol O-acyltransferase (SOAT1, previously called ACAT1) present in splenic macrophages, steroidogenic organs, kidneys, and sebaceous glands [21–23]. The other enzyme is SOAT2 (previously called ACAT2) which is expressed in enterocytes and hepatocytes [22–25]. The tissue distribution and functions of SOAT1 and 2 are discussed in a 2012 review [26]. Ordinarily in most tissues, with the exception of organs like the adrenal gland, only a small proportion of the cholesterol present is esterified [27,28]. However, tissue EC levels can rise appreciably under various conditions as seen for example in the liver during periods of high dietary cholesterol intake [29]. Esterified cholesterol can also become greatly elevated in rare diseases such as Tangier disease [30], Wolman disease (WD) and Cholesteryl Ester Storage Disease (CESD) [31]. In a mouse model of CESD the elimination of SOAT2 resulted in a marked reduction in EC sequestration in the liver and small intestine [32]. Other studies demonstrated that in *Npc1^{-/-}* mice lacking SOAT2 there was less hepatomegaly and UC sequestration, and lower liver transaminase levels [33]. In these models the elimination of SOAT2-driven EC formation likely diminished the EC content of nascent very low density lipoproteins (VLDL) and chylomicrons to the extent that the remnants of these particles ultimately cleared from the circulation carried less cholesterol for potential sequestration in the E/L compartment because of mutations in *LIPA*, *NPC1* or *NPC2*.

In the formative studies that explored in vivo the impact of acute 2HP β CD treatment on cholesterol metabolism in *Npc1^{-/-}* mice, the most striking changes seen 24 h later occurred in the liver and spleen where a dramatic drop in the UC level was accompanied by a decisive rise in tissue EC concentration, together with a profound fall in the rate of cholesterol synthesis [34–37]. However, it has not been determined whether in the liver and spleen, or indeed in most organs, these two adaptive mechanisms together are largely responsible for offsetting the release of entrapped UC over the first 24 h after treatment starts. To address this question two sets of young adult mice of four genotypes (*Npc1^{+/+}* : *Soat2^{+/+}*, *Npc1^{+/+}* :

Soat2^{-/-}, *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-}) that had 24 h earlier received a bolus subcutaneous injection of 2HPβCD in saline, or saline only, were used for the measurement of either UC and EC concentrations in liver and spleen, or the absolute rate of sterol synthesis in these organs. The data show that in the livers of the treated *Npc1*^{-/-} : *Soat2*^{+/+} mice the defensive changes in EC formation and cholesterol synthesis together amounted to 64% of the UC mass that was mobilized during the 24 h after 2HPβCD administration. In the treated *Npc1*^{-/-} : *Soat2*^{-/-} mice, where the pre-treatment hepatic levels of UC were appreciably less than in their *Npc1*^{-/-} : *Soat2*^{+/+} counterparts, almost no new EC was generated but the adaptive fall in cholesterol synthesis equated to 85% of the mass of UC that was liberated. A very different result was seen in the spleen where the corresponding figures for the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice were only 13% and 23%, respectively. These findings, especially for the liver may have particular relevance to the actions of systemically administered 2HPβCD in NPC1 patients given there is evidence that the level of hepatic SOAT2 activity in humans generally may be far lower than it is in mouse models.

2. Materials and methods

2.1 Animals

Npc1^{+/-} mice (pure BALB/c) originally obtained from a colony at the National Institute of Neurological Diseases and Stroke [38] were source of the NPC1 mutation (*Npc1*^{-/- nih}) used in this project. *Soat2*^{+/-} mice (mixed 129Sv : C57BL/6 background) were purchased from Jackson Laboratories. Thus in the current studies all mice were littermates of a mixed strain background (BALB/c : 129Sv : C57BL/6) derived from *Npc1*^{+/-} : *Soat2*^{+/-} breeding stock as described previously [33]. This resulted in progeny of multiple genotypes, four of which were selected for study. These were *Npc1*^{+/+} : *Soat2*^{+/+}, *Npc1*^{+/+} : *Soat2*^{-/-}, *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-}. The pups were weaned at 21 days on to a low-cholesterol, cereal-based, chow diet with a fat content of ~4% (No. 7001, Envigo :Teklad, Madison, WI). The mice were group housed in plastic colony cages containing wood shavings, and had continual access to drinking water. They were kept in a light-cycled room with 12 h of light (1200–2400 h) and darkness (0–1200 h). All mice were studied in the fed state at about 1100h. The studies were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory Animals, and the protocols used were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

2.2 Administration of 2HPβCD

A 20% (w/v) solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich product H107) was prepared by dissolution in sterile saline at room temperature. During the late dark phase the mice were administered a single subcutaneous injection of 2HPβCD at the scruff of the neck. The dose was 4000 mg/kg body weight. Matching mice injected with saline only served as controls. After injection all mice were returned to their cages until used for metabolic measurements 24 h later. Two sets of mice were established, one for the measurement of tissue UC and EC concentrations and the other for determining absolute rates of sterol synthesis in the liver and spleen. The small intestines were also taken from

these mice for such measurements as described in an earlier publication [39]. All groups contained a varying proportion of male and female mice as noted in Table 1.

2.3 Esterified and unesterified cholesterol concentrations

Approximately 24 h after they had received their respective treatments the mice were anesthetized and exsanguinated from the inferior vena cava, with 0.8 to 1.0 ml of blood being obtained. The liver and spleen were excised, rinsed in cold saline, blotted and weighed. Aliquots of liver totaling 300 to 350 mg and the whole spleen were added to ~40 ml of chloroform:methanol (2:1 v/v). The absolute concentrations of esterified and unesterified cholesterol were measured by established methods using a combination of column and gas chromatography [33]. The concentrations were expressed as mg/g wet tissue weight. No corrections were made for the presence of EC and UC contained in residual blood in the tissue aliquots. In addition, as all mice were in the fed state at exsanguination, plasma total cholesterol concentrations and lipoprotein compositions were not determined.

2.4 Cholesterol synthesis rates

The cholesterol synthesis measurements were made towards the end of the dark phase over a 1-h period starting 23 h after the 2HP β CD, or saline only, had been administered. Custom-generated tritiated water, with an activity of 5 Ci/ml (PerkinElmer Life Sciences, Waltham, MA) was diluted in sterile saline to an activity of approximately 200 mCi/ml. The mice were weighed, given an intraperitoneal injection of [³H]water (~2 mCi/g body weight), and then kept in a warm environment under a well ventilated fume hood for the next 60 min. They were then anesthetized and exsanguinated into a heparinized syringe. The liver and spleen were excised, rinsed in cold saline, blotted and weighed. The liver was cut into multiple pieces and an aliquot (~300–350 mg) was placed in 5 ml of ethanolic KOH as was the entire spleen. From each mouse, a 100 μ l aliquot of plasma was taken through a series of dilutions for the measurement of the plasma water specific activity. The labeled sterols in the digested tissues were extracted and quantitated [40]. The rate of cholesterol synthesis was expressed in two ways. The first was the nmol of water incorporated into sterol per hour per gram wet weight of tissue (nmol/h/g). These data, which did not include a correction for the presence of labeled sterol in the residual blood present in the tissues were subsequently used to calculate the absolute quantity of sterol produced over 24 h (mg of sterol synthesized/day/g). The terms used in the equation to make this calculation are defined elsewhere [41,42]. It should be emphasized that digitonin precipitates not only cholesterol but also several of the intermediates in the biosynthetic pathway including desmosterol, lathosterol and lanosterol [43]. Their respective positions in the synthetic pathway are well documented [44].

2.5 Analysis of data

All values are presented as the mean \pm SEM of data from the specified number of animals. GraphPad Prism 6.02 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. Differences between means were tested for significance ($p < 0.05$) using a one-way analysis of variance (ANOVA) and applying Tukey's multiple comparison test.

3. Results

3.1. In the livers of the *Npc1*^{-/-} : *Soat2*^{+/+} mice the reduction in UC levels induced by 2HPβCD resulted in both a decisive rise in tissue EC levels and a marked suppression of sterol synthesis but in their *Npc1*^{-/-} : *Soat2*^{-/-} counterparts only the latter response occurred

In reviewing the organ weight data in Table 1 it should be noted that the reduction in hepatomegaly but not splenomegaly in *Npc1*^{-/-} : *Soat2*^{-/-} mice has been previously documented [31]. Those earlier studies also showed that the absence of SOAT2 alone caused a marked reduction in hepatic EC levels in mice with or without NPC1. The data from the new studies demonstrate how much SOAT2 function influences the impact of acute 2HPβCD treatment on hepatic cholesterol levels and rates of synthesis in mice lacking NPC1. The concentration of UC in the *Npc1*^{-/-} : *Soat2*^{+/+} fell on average by 5.8 mg/g over 24 h (Fig. 1A). In the matching *Npc1*^{-/-} : *Soat2*^{-/-} mice where there was less UC entrapment at baseline the reduction was 2.6 mg/g ($p > 0.05$). Even more striking are the data for hepatic EC levels, particularly in the treated *Npc1*^{-/-} : *Soat2*^{-/-} vs *Npc1*^{-/-} : *Soat2*^{+/+} mice (Fig. 1B). Consistent with the results of past studies the mice deficient in NPC1 alone exhibited the prototypical marked increase in hepatic EC levels. In the matching treated *Npc1*^{-/-} : *Soat2*^{-/-} mice EC levels did not change. The rates of hepatic sterol synthesis, measured in vivo in a different set of mice, are presented in Fig. 1C. Here the two most notable findings pertain to the mice lacking NPC1, with and without SOAT2. One is that the rate of hepatic sterol synthesis at baseline in the *Npc1*^{-/-} : *Soat2*^{-/-} mice was significantly higher than in their *Npc1*^{-/-} : *Soat2*^{+/+} counterparts. The other is that despite this difference 2HPβCD treatment resulted in a profound reduction in the rate of synthesis in both types of mice.

3.2. In the spleens of both the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice treatment with 2HPβCD led to increased cholesterol esterification as well as suppression of sterol synthesis

The splenomegaly seen in the *Npc1*^{-/-} : *Soat2*^{+/+} mice was equally evident in their *Npc1*^{-/-} : *Soat2*^{-/-} littermates and was not impacted by 2HPβCD treatment in either case (Table 1). This treatment also did not change the concentrations of UC or EC in the spleens of either the *Npc1*^{+/+} : *Soat2*^{+/+} or *Npc1*^{+/+} : *Soat2*^{-/-} mice (Figs 2A and 2B, respectively). However, there was a decisive reduction in the UC concentration after 2HPβCD was given in both the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice (Fig. 2A). The splenic EC concentrations in these same two groups of mice increased markedly and to about the same degree after 2HPβCD administration (Fig 2B). The pattern of genotypic and treatment-related changes in cholesterol synthesis rates in the spleen (Fig. 2C) paralleled those found in the liver (Fig. 1C), with the main finding being a profound reduction in cholesterol synthesis rates in both the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice in response to 2HPβCD.

3.3. The quantitative importance of cholesterol synthesis suppression either alone or with a concomitant rise in cholesterol esterification in countering the 2HP β CD-driven release of sequestered UC was much greater in the liver than in the spleen

The objective of Fig. 3 is to present a side-by-side comparison of how much an increase in SOAT2-driven cholesterol esterification on the one hand and a suppression of local cholesterol synthesis on the other compare quantitatively in accommodating the 2HP β CD-mediated release of UC in the liver and spleen over the 24 h following its acute administration. These data are presented for just the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice because 2HP β CD treatment did not impact UC levels in the corresponding groups that were *Npc1*^{+/+}. The magnitude of the drop in UC levels in both organs after 2HP β CD was less pronounced in the *Npc1*^{-/-} : *Soat2*^{-/-} mice than in their *Npc1*^{-/-} : *Soat2*^{+/+} counterparts, especially in the liver (Fig. 3A). This was not unexpected given that *Npc1*^{-/-} mice without SOAT2 have lower baseline levels of UC especially in the liver. The data in Fig. 3B are particularly striking. For the liver there was a substantial rise in hepatic EC levels in the *Npc1*^{-/-} : *Soat2*^{+/+} mice after treatment. This accounted for about 38% of the fall in UC after 2HP β CD was given. In the matching *Npc1*^{-/-} : *Soat2*^{-/-} mice essentially none of the released UC underwent esterification. The data for the spleen sharply contrast with those for the liver. Thus in both the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice there were comparable but small increments in EC levels in spleen after 2HP β CD. In the case of the *Npc1*^{-/-} : *Soat2*^{+/+} mice the rise in EC levels accounted for only about 7% of the amount of UC lost from the spleen over the 24-h period. For their treated *Npc1*^{-/-} : *Soat2*^{-/-} littermates this figure was about 10%.

The data in Fig. 3C similarly show a decisive difference between the two organs in the extent to which adaptive reductions in the absolute amount of cholesterol synthesized offset the release of UC after 2HP β CD administration. In the livers of the *Npc1*^{-/-} : *Soat2*^{+/+} mice the reduction of 1.41 mg/g in newly synthesized sterol over 24 h equated to 24% of the 5.78 mg/g of UC released. When combined with the amount of released UC that was esterified in these particular mice over this time (2.19 mg/g, which was equal to 38% of the drop in the UC level) it is clear that together these two mechanisms represented the primary defense against the surge in unsequestered UC after the 2HP β CD was given. For their *Npc1*^{-/-} : *Soat2*^{-/-} counterparts the reduction of 2.16 mg/g in the amount of sterol synthesized by the liver over 24 h equated to 83% of the 2.60 mg/g fall in the UC level. There was a negligible rise in hepatic esterification of UC in these mice thus making sterol synthesis inhibition the overwhelming defensive mechanism in the absence of SOAT2-mediated esterification.

In marked contrast to what occurred in the livers, in the spleens of both the *Npc1*^{-/-} : *Soat2*^{+/+} and *Npc1*^{-/-} : *Soat2*^{-/-} mice the pronounced reduction in sterol synthesis over the 24-h post treatment period represented, in absolute terms, only a minor adaptive response to the substantial amounts of UC released after 2HP β CD administration. Here it must be noted however that the rate of sterol synthesis in the spleen across all genotypes and treatments was much lower than seen for the livers in these same animals. Thus in the spleens of the *Npc1*^{-/-} : *Soat2*^{+/+} mice the 2HP β CD-facilitated release of 5.09 mg of UC /g resulted in a fall in cholesterol synthesis of just 0.31 mg/day/g, along with a rise in EC formation amounting to only 0.38 mg/g. Together, these adaptive changes were equivalent to only 13%

of the amount of UC released after 2HP β CD was given. In the spleens of their treated *Npc1^{-/-} : Soat2^{-/-}* counterparts, the liberation of 3.98 mg of UC/g was offset by a reduction in sterol synthesis of only 0.42 mg/day/g and an increment in the EC level of just 0.50 mg/g. The sum of these defensive changes combined represented only 23% of the amount of UC level liberated by 2HP β CD. Taken together these various sets of data show that at least in this *Npc1^{-/-}* mouse model the 2HP β CD-facilitated release of UC in the spleen is offset primarily through a mechanism(s) of greater quantitative importance than that derived from a combination of cholesterol synthesis inhibition and increased cholesterol esterification.

4. Discussion

The impetus for the development of 2HP β CD as a therapy for NPC1 disease derives from a plethora of data generated in studies using mouse and cat models with varying mutations in *NPC1* [12,13,45]. The first clinical trial investigated the impact of intrathecal delivery of 2HP β CD on neurological disease progression [16]. Several other trials, either at or near completion ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02939547) Identifier [NCT02939547](https://clinicaltrials.gov/ct2/show/study/NCT02939547) and [NCT02912793](https://clinicaltrials.gov/ct2/show/study/NCT02912793)) or currently enrolling patients ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03887533) Identifier [NCT03887533](https://clinicaltrials.gov/ct2/show/study/NCT03887533)), have or will evaluate repeated systemic administration of 2HP β CD on multiple parameters including specific indices of hepatic cholesterol metabolism in NPC1 patients over a broad age range. Questions will nevertheless remain concerning the relative quantitative importance of cholesterol synthesis suppression and increased esterification as first-line defense mechanisms in protecting the liver and indeed other organs like the spleen against the toxic effects of a surge in cellular UC content caused by high doses of systemically administered 2HP β CD. It is important to note here that while the *Npc1^{-/- nih}* model has been widely used, newer models have been developed including the *Npc1^{I1061T}* mouse which expresses the most common human *NPC1* mutation [46], and more recently the *Npc1^{em1Pav}* mouse [47]. A comprehensive review of animal models for NPC disease research appeared in 2019 [10]. The current and earlier studies on the potential benefit of eliminating SOAT2 function in NPC1 disease used the *Npc1^{-/- nih}* model mainly because its cholesterol metabolism has been characterized at a biochemical and molecular level far more extensively than is the case for other murine models with disrupted NPC1 function.

In evaluating the findings presented here several points should be made regarding potential sources of error in the measurements and the conclusions drawn from them. The first of these pertains to two aspects of the sterol synthesis data. These were measured as the rates of incorporation of water into newly generated sterol over a 1-h interval corresponding to the 24th hour after the acute administration of 2HP β CD or saline alone. These rates were subsequently converted to an absolute amount of sterol synthesized over 24 h using established equations (Fig. 3C). Thus the question this calculation raises is whether the rate measured in the 24th hour faithfully reflected the amount of sterol generated during the course of the preceding 23 h given that hepatic sterol synthesis rates usually show a marked diurnal variation [48,49]. In our early studies with *Npc1^{-/-}* mice we found that, unlike their *Npc1^{+/+}* littermates, they did not exhibit diurnal changes in their rate of hepatic sterol synthesis [50]. The second point to consider in evaluating the sterol synthesis data is that past studies showed the suppressive impact of the liberated UC on the biosynthetic pathway in this model does not become maximal in either the liver or spleen until 3 to 6 h after the

2HP β CD is administered [37]. Thus the conversion of rates of incorporation of labeled water into new sterol measured in the 24th hour following treatment (Fig. 1C and 2C) into absolute amounts of sterol synthesized over 24 h (Fig. 3C) results in an overestimation of the true amount of new sterol generated per day. The magnitude of this error, which is likely to be small, would be the same for both the liver and spleen.

With regard to the tissue UC and EC data one potential source of error warranting comment relates to the presence of UC and EC in residual blood within the tissue aliquots taken for analysis. Earlier studies using ⁵⁹Fe to assess residual tissue blood content (% of wet weight) in multiple organs of adult C57BL6 mice after 400ul of blood was removed found values for the spleen and liver of 22.1 and 5.3%, respectively [51]. Whole blood contains UC in cell membranes as well as varying levels of UC and EC in lipoproteins in the plasma compartment, with this EC having been generated mainly through the actions of SOAT2 and also of lecithin-cholesterol acyltransferase (LCAT) in plasma [52]. Given that in the current studies the amount of blood removed at exsanguination was at least 0.8 ml across all experimental groups, the degree of error stemming from residual blood contamination is believed to not significantly impact the calculations for the treatment-driven changes in UC or EC levels for either organ in the *Npc1*^{-/-} : *Soat2*^{+/+} mice and their *Npc1*^{-/-} : *Soat2*^{-/-} counterparts (Fig. 3A and 3B). Perhaps the most striking point about the UC concentration data is that even though the baseline levels in the spleen of the *Npc1*^{-/-} : *Soat2*^{+/+} mice (Fig. 2A) were lower than those for the liver (Fig. 1A), the magnitude by which they fell in the 24 h after 2HP β CD administration was about the same in both organs (Fig. 3A). In the case of the matching *Npc1*^{-/-} : *Soat2*^{-/-} mice the extent of the fall in the liver UC levels after 2HP β CD treatment was not as much as that for the spleens (Fig. 3A). This reflected the fact that the absence of SOAT2 in itself drove down baseline UC levels in the livers but not the spleens of *Npc1*^{-/-} mice. In regards to the EC data for the liver (Fig. 1B) it should be noted that past studies demonstrated high levels of SOAT2 activity in the livers of chow-fed mice of a mixed background (C57BL/6 : 129Sv) mice [53]. Thus the marked rise in EC levels in the 24 h after 2HP β CD administration seen in the *Npc1*^{-/-} : *Soat2*^{+/+} mice was anticipated. However, the finding that hepatic EC levels in the matching treated *Npc1*^{-/-} : *Soat2*^{-/-} mice did not change might be considered surprising given that macrophage presence increases in several organs including the liver when NPC1 function is disrupted [54]. Although our past studies in *Npc1*^{-/-} : *Soat2*^{-/-} mice showed less of an increase in hepatic mRNA levels for SOAT1 than seen in their *Npc1*^{-/-} : *Soat2*^{+/+} counterparts, they were still appreciably elevated compared to those found in matching *Npc1*^{+/+} : *Soat2*^{+/+} and *Npc1*^{+/+} : *Soat2*^{-/-} mice [33]. One might speculate therefore that in the *Npc1*^{-/-nih} mouse model the proportion of total hepatic cholesterol esterifying activity attributable to SOAT1 is just a fraction of that occurring via SOAT2 as has been reported for the African Green monkey [25]. Irrespective of how much of the liberated UC is esterified by each of these enzymes, ultimately the hepatic pool of EC is hydrolyzed with some, if not most, of the resulting UC appearing in the bile and later the fecal neutral sterol fraction [35,55].

In addition to esterification another potential mechanism for rapid handling of the liberated UC, particularly in the absence of SOAT2, might be its ABCA1-mediated efflux onto ApoA1 to form nascent HDL. Studies by Pedrelli et al. using mice injected with an antisense oligonucleotide targeted to hepatic SOAT2 detected increased hepatic ABCA1 protein levels

[56]. However, a study by Taylor et al. using *Npc1^{-/-nih}* mice given 2HP β CD found hepatic ABCA1 mRNA levels to be mostly unchanged except for a transient rise 6 h after treatment [37]. This was attributed to ABCA1 not being an LXR target gene in hepatocytes where a specific ABCA1 promoter is required.

Irrespective of the comparative levels of SOAT1 and 2 activity in the liver of the *Npc1^{-/- nih}* mouse and other murine models of NPCD, the EC data for the spleen (Fig.2B) clearly show that 2HP β CD administration stimulated esterification of UC in this organ in the absence of SOAT2 thus confirming a significant presence of SOAT1. This finding is consistent with data showing a comparatively high mRNA expression level for SOAT1 in human spleen [23]. The more striking finding for the spleen in the current studies was that the 2HP β CD-driven release of UC was offset to only a modest degree through the combined defensive actions of increased esterification and suppression of synthesis of cholesterol (Fig. 3B and 3C). In the studies of Taylor et al. a marked and sustained increase in mRNA levels for ABCA1 in the spleen was found as early as 2 h after 2HP β CD administration [37]. Thus it is attractive to speculate from those data that ABCA1-mediated efflux of UC represents a quantitatively important pathway for removing liberated UC from the spleen in concert with suppression of synthesis and raised esterification rates of cholesterol.

A major question arising from the data presented here is their relevance to hepatic cholesterol metabolism in NPC1 patients before and following systemic 2HP β CD treatment, with a focus on SOAT2-driven cholesterol esterification. It is important to first point out what is known about hepatic cholesterol levels in patients with either NPC1 or NPC2 disease. There appear to be no published data specifically for hepatic UC and EC levels in either NPC1 or NPC2 patients. However, more than a decade before the discovery of mutations in NPC2 Vanier reported total cholesterol levels in the livers and spleens of patients with NPC disease [57]. For a total 15 NPC patients and 6 control subjects, hepatic total cholesterol concentrations (mean \pm SEM) of 9.7 ± 0.6 and 4.6 ± 0.5 mg/g wet weight, respectively, were found. The latter value is consistent with data from two other studies that reported the total cholesterol concentration in normal human liver to be 3.9 ± 0.2 mg/g (n=12) [58] and 5.4 ± 1.1 mg/g (n=11) [59], respectively. In one of those studies the fraction of cholesterol present in the esterified form averaged 25% [58]. While further data for hepatic cholesterol levels in NPC1 patients might arise from future studies, it is interesting that the values found in the Vanier study are appreciably less than those seen in animal models of NPC1 disease that were maintained on low-cholesterol rodent chow diets. This is best exemplified by data for untreated adult *Npc1^{-/-nih}* mice on a pure BALB/c background where total cholesterol levels in liver were generally in the range of ~20–30 mg/g which represented at least a 7-fold increase over those found in matching wild-type controls [60–62]. This remained the case even in the mixed strain *Npc1^{-/-} : Soat2^{+/+}* mice in the present studies (Fig. 1A). Comparable findings have also been reported for the *Npc1^{nmt164}* model on a pure C57BL/6 background [63]. It is also noteworthy that early research using a feline model of NPC1 disease found hepatic total cholesterol concentrations of ~18 and ~2.3 mg/g, respectively in the mutants and normal controls [64].

Such comparative data raise many more questions than can be addressed here. One has to do with the link between SOAT2-driven esterification of cholesterol in enterocytes and

hepatocytes and the levels of hepatic UC entrapment resulting from NPC1 dysfunction. The amount of lipoprotein cholesterol cleared daily from the circulation by the liver far exceeds that of any other organ. Thus not only is it the site of clearance of chylomicron remnants containing cholesterol absorbed from the small intestine [65] but it ordinarily is also the principal organ for removing LDL and related apoB-containing lipoproteins from the circulation [66]. Hypothetically, when NPC1 function is impaired or lost, the concurrent elimination of SOAT2 activity by either genetic or pharmacological manipulation should lower the level of hepatic UC sequestration because less EC would be generated in hepatocytes for incorporation into nascent VLDL and ultimately mature LDL particles. This should in turn result in lower levels of UC entrapment when these particles are cleared from the circulation. Such a scenario is borne out by the lower hepatic UC levels and reduced plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in *Npc1^{-/-} : Soat2^{-/-}* vs *Npc1^{-/-} : Soat2^{+/+}* mice [33]. It must be emphasized however that although it is widely agreed there is a high level of expression of SOAT2 in the small intestine of humans [23,24], a nonhuman primate model [21], and mice of a mixed genetic background (C57BL/6 : 129 Sv) [53], available data suggest that SOAT2 activity in human hepatocytes is very low [25] compared to that detected in the livers of a primate model [25] and of C57BL/6 : 129Sv mice [53]. Even if this were not the case the selective and potent inhibitors of SOAT2 that have a decisive therapeutic benefit in mouse models of CESD [67] and atherosclerosis [68] have not yet been approved for use in humans. This raises the question of what other type of agent that targets cholesterol handling by the small intestine and subsequently the liver could serve as an adjunct to systemic 2HPβCD administration aimed primarily at ameliorating liver disease in specific NPC1 patients. Interest in this question is driven partly by the results of studies in humans [69,70] and a feline NPC1 model [15] indicating adverse effects of high doses of systemic 2HPβCD administration on the lungs. One possible candidate for combination therapy is ezetimibe, a potent inhibitor of cholesterol absorption [71] which is FDA-approved for use in pediatric patients with heterozygous familial hypercholesterolemia who are 10 years and older [72]. Ezetimibe also has been shown to reduce the severity of liver disease not only in the *Npc1^{-/- nih}* mouse [73] but also in a mouse model for CESD [74] as well as in patients with CESD [75].

5. Conclusion

The sequestration of unesterified cholesterol (UC) in tissues resulting from mutations in the NPC1 gene can be overcome by acute systemic treatment with 2-hydroxypropyl-β-cyclodextrin (2HPβCD). However, compensatory mechanisms are needed to offset the resulting surge in cellular UC content which can be cytotoxic. In the current studies using NPC1-deficient mice, with and without the cholesterol esterifying enzyme SOAT2, it was found that in the liver but not the spleen the suppression of new cholesterol synthesis with or without increased esterification provided the major immediate defenses against the 2HPβCD-facilitated release of UC. This was not the case in the spleen where we conclude cholesterol transporters may have facilitated the cellular exit of much of this excess UC.

Acknowledgements

This research was supported by National Institutes of Health Grants R01 HL 009610 (SDT) and DK 078592 (JJR) and the Ara Parseghian Medical Research Foundation (JJR). Dr. Turley also received partial salary support from the Department of Internal Medicine at UT Southwestern. We thank the late Dr. Lawrence Rudel of Wake Forest University School of Medicine for informative discussions about possible species differences in the activities of cholesterol esterifying enzymes in various organs. The assistance of Monti Schneiderman with the maintenance of the mouse colony is gratefully acknowledged.

Abbreviations

ABCA1	ATP-binding cassette transporter A1
bw	body weight
EC	esterified cholesterol
E/L	endosomal / lysosomal
2HPβCD	2-hydroxypropyl- β -cyclodextrin
LAL	lysosomal acid lipase
NPC1	Niemann-Pick type C1
NPC2	Niemann-Pick type C2
SOAT1	sterol O-acyltransferase 1
SOAT2	sterol O-acyltransferase 2
UC	unesterified cholesterol

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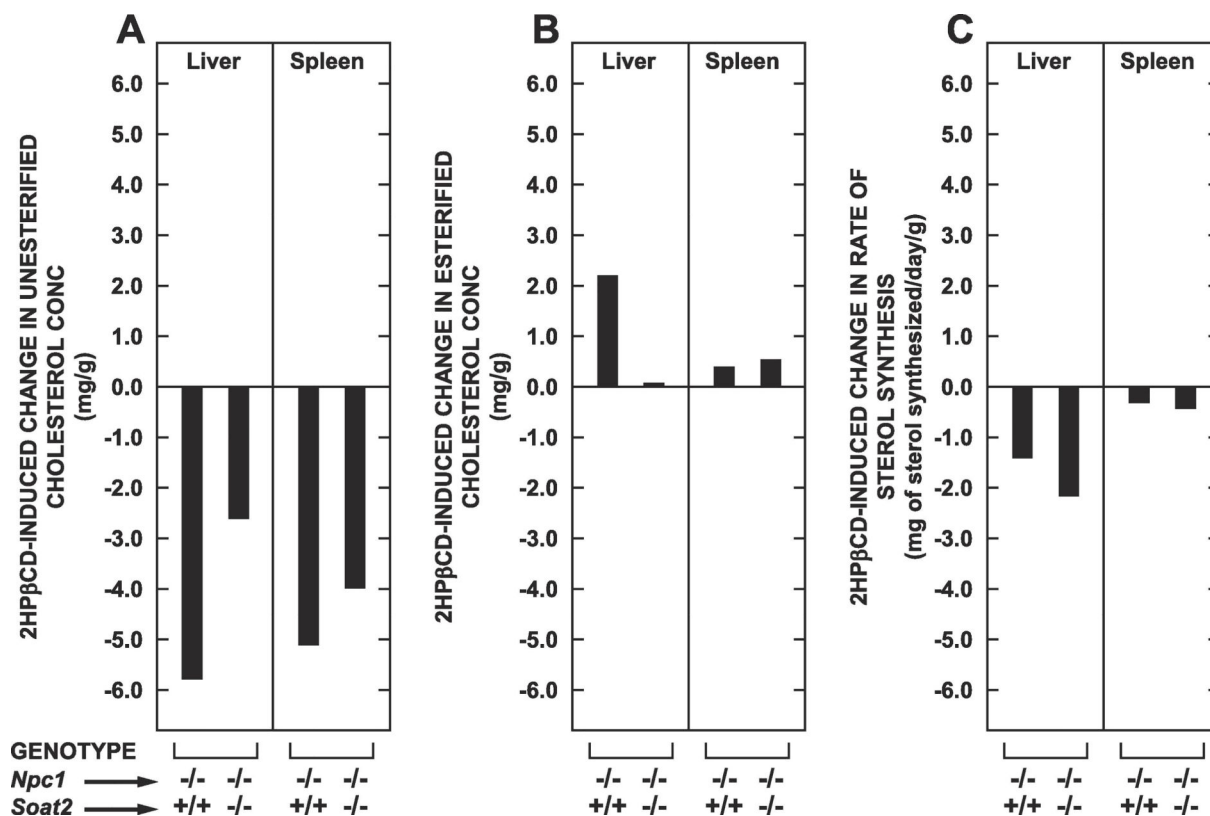
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Highlights

- NPC1 is a cholesterol transporter in the lysosomal/endosomal compartment of cells
- NPC1 deficiency causes entrapment of unesterified cholesterol (UC) and other lipids
- 2-hydroxypropyl- β -cyclodextrin releases the sequestered UC which can be cytotoxic
- Adaptive responses include less synthesis and more esterification of UC
- In *Npc1*^{-/-} mice these are the major adaptations in the liver but not the spleen

**Fig. 1.**

In the livers of *Npc1*^{-/-} ; *Soat2*^{+/+} mice the 2HPβCD-induced release of entrapped UC resulted in both increased EC formation along with marked inhibition of cholesterol synthesis whereas in their *Npc1*^{-/-} ; *Soat2*^{-/-} counterparts only the latter response was seen. For the study in which the data for tissue UC and EC concentrations (panels A and B, respectively) were obtained there were 6 mice per group. The sterol synthesis measurements (panel C) were made in separate mice with 6 per group in all cases except for the *Npc1*^{+/+} ; *Soat2*^{-/-} mice where there were 4 given either saline alone or 2HPβCD in saline. Values are the mean ± SEM. Different letters denote statistically significant ($p < 0.05$) differences as determined by one-way ANOVA.

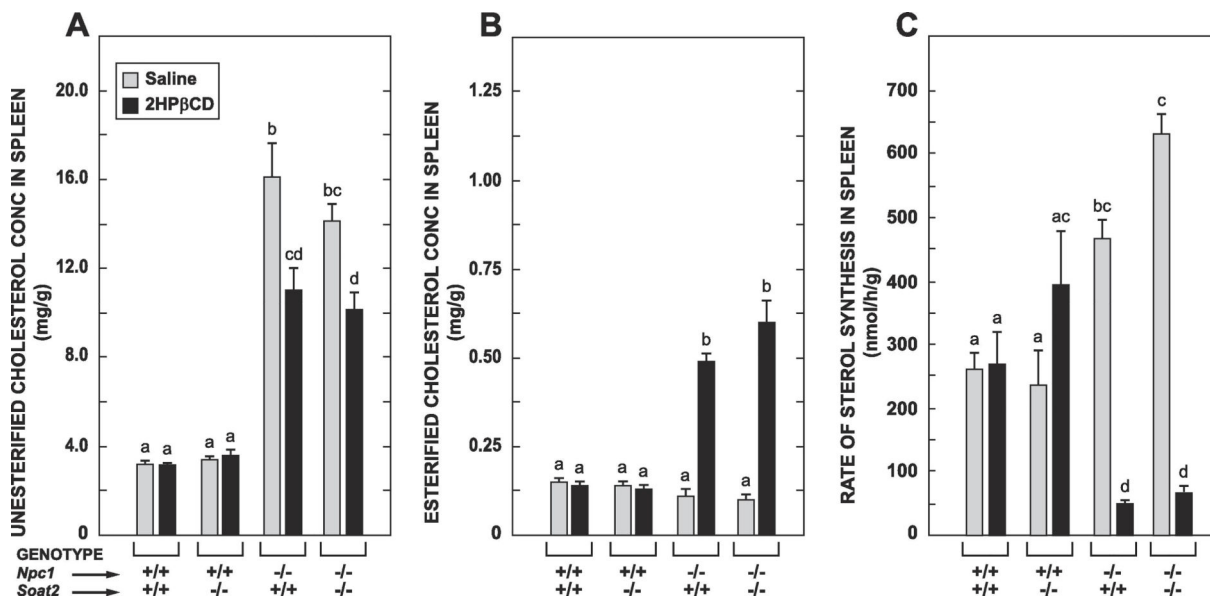


Fig. 2.

In the spleens of both the $Npc1^{-/-}; Soat2^{+/+}$ and $Npc1^{-/-}; Soat2^{-/-}$ mice the 2HPβCD-induced reduction in UC levels resulted in comparable increments in rates of cholesterol esterification as well as similar degrees of cholesterol synthesis inhibition. The data in panels A-C were obtained from the same respective sets of mice used for the corresponding measurements in the liver. Values are the mean \pm SEM. Different letters denote statistically significant ($p < 0.05$) differences as determined by one-way ANOVA.

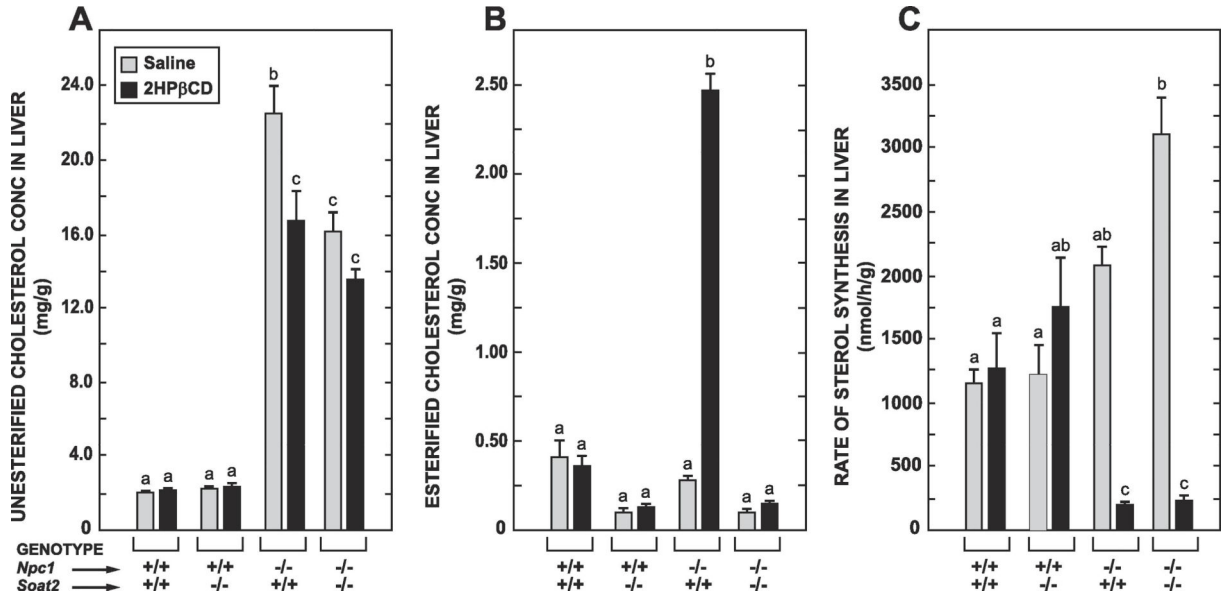


Fig. 3. Comparison of the net change in UC concentration with that in the EC concentration and the absolute rate of sterol synthesis in the liver and spleen of *Npc1*^{-/-} : *Soat2*^{+/+} versus *Npc1*^{-/-} : *Soat2*^{-/-} mice in the 24-h period following acute 2HPβCD treatment. Corresponding data for the matching *Npc1*^{+/+} : *Soat2*^{+/+} and *Npc1*^{+/+} : *Soat2*^{-/-} mice are not shown because 2HPβCD treatment did not result in statistically significant changes in any of the three parameters measured in either the liver (Fig. 1 A–C) or spleen (Fig. 2 A–C). The rates of sterol synthesis shown in panel C of both Fig 1 and Fig 2 represent the nmol of water incorporated into sterol per g of wet weight tissue per h. As described in Materials and Methods, these rates were converted to an approximate absolute amount of sterol generated, with the units of mg synthesized per day per g wet weight of tissue. Then, as was the case for estimating the net change in UC and EC levels following 2HPβCD treatment (panels A and B, respectively), the magnitude of the net change in sterol synthesis (panel C) was determined as the difference in the mean values between the 2HPβCD- and saline only-treated groups.

Table 1

Body and organ weights of mice deficient in either NCP1 or SOAT2, or both genes, that were given a single treatment with 2-hydroxypropyl- β -cyclodextrin in saline, or saline alone 24h before study

Genotype	Treatment	No. of mice	Body weight	Liver weight	Spleen weight
		♂ / ♀	(g)	(g)	(g)
<i>Npc1^{+/+} : Soat2^{+/+}</i>	Saline	7 / 5	23.1 ± 1.0	1.29 ± 0.08 ^a	0.067 ± 0.003 ^a
<i>Npc1^{+/+} : Soat2^{+/+}</i>	2HP β CD	7 / 5	23.6 ± 1.2	1.32 ± 0.09 ^a	0.077 ± 0.003 ^a
<i>Npc1^{+/+} : Soat2^{-/-}</i>	Saline	5 / 5	21.7 ± 1.2	1.18 ± 0.09 ^a	0.068 ± 0.003 ^a
<i>Npc1^{+/+} : Soat2^{-/-}</i>	2HP β CD	5 / 5	22.1 ± 1.4	1.14 ± 0.07 ^a	0.064 ± 0.004 ^a
<i>Npc1^{-/-} : Soat2^{+/+}</i>	Saline	7 / 5	20.7 ± 1.0	1.79 ± 0.07 ^b	0.107 ± 0.007 ^b
<i>Npc1^{-/-} : Soat2^{+/+}</i>	2HP β CD	8 / 4	20.8 ± 1.0	1.76 ± 0.10 ^b	0.114 ± 0.009 ^b
<i>Npc1^{-/-} : Soat2^{-/-}</i>	Saline	7 / 5	19.7 ± 0.8	1.43 ± 0.10 ^a	0.113 ± 0.010 ^b
<i>Npc1^{-/-} : Soat2^{-/-}</i>	2HP β CD	8 / 4	20.8 ± 1.0	1.56 ± 0.11 ^{a,b}	0.137 ± 0.017 ^b

All mice were 48–51 days old at the time of study. Approximately one half of the mice in each group were used for tissue cholesterol concentration measurements. The remaining mice were used specifically for determining rates of cholesterol synthesis in vivo. Values are mean \pm SEM for the total number of mice indicated. Different letters denote statistically significant differences as determined by one-way ANOVA using Tukey's multiple comparison test.