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Ontogenic changes in lung cholesterol metabolism, lipid content, and histology in mice with Niemann–Pick type C disease

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ABSTRACT

Niemann–Pick Type C (NPC) disease is caused by a deficiency of either NPC1 or NPC2. Loss of function of either protein results in the progressive accumulation of unesterified cholesterol in every tissue leading to cell death and organ damage. Most literature on NPC disease focuses on neurological and liver manifestations. Pulmonary dysfunction is less well described. The present studies investigated how Npc1 deficiency impacts the absolute weight, lipid composition and histology of the lungs of *Npc1*$^{-/-}$ mice (*Npc1*^{nih}) at different stages of the disease, and also quantitated changes in the rates of cholesterol and fatty acid synthesis in the lung over this same time span (8 to 70 days of age). Similar measurements were made in *Npc2*$^{-/-}$ mice at 70 days. All mice were of the BALB/c strain and were fed a basal rodent chow diet. Well before weaning, the lung weight, cholesterol and phospholipid (PL) content, and cholesterol synthesis rate were all elevated in the *Npc1*$^{-/-}$ mice and remained so at 70 days of age. In contrast, lung triacylglycerol content was reduced while there was no change in lung fatty acid synthesis. Despite the elevated PL content, the composition of PL in the lungs of the *Npc1*$^{-/-}$ mice was unchanged. H&E staining revealed an age-related increase in the presence of lipid-laden macrophages in the alveoli of the lungs of the *Npc1*$^{-/-}$ mice starting as early as 28 days. Similar metabolic and histologic changes were evident in the lungs of the *Npc2*$^{-/-}$ mice. Together these findings demonstrate an intrinsic lung pathology in NPC disease that is of early onset and worsens over time.

1. Introduction

In Niemann–Pick type C (NPC) disease unesterified cholesterol (UC), gangliosides, and glycosphingolipids continually accumulate in the late endosomal/lysosomal (E/L) compartment of all cells in every organ. It is an autosomal recessive disorder primarily affecting children and is characterized by neurodegeneration, hepatic and pulmonary dysfunction, and premature death [1]. A lot of what we currently know about NPC1 and NPC2 function, and also the etiology and pathogenesis of NPC disease has come from the study of animal models with NPC1 or NPC2 deficiency, primarily in the mouse [2–14], and also from various in vitro systems as well as techniques such as X-ray crystallography [15–17]. Unequivocally, it is the sequestration of UC in the E/L compartment that is the fundamental cause of NPC disease [18]. This accumulation of UC is due to a loss of function mutation in genes encoding either NPC1 or NPC2, both of which are essential for facilitating cholesterol transport out of the late E/L compartment [17]. Disruption of this egress and the resultant buildup of UC, together with that of other types of lipids, leads to cell death and multi-organ damage. While neurodegeneration is a major cause of morbidity and mortality in NPC disease, there are cases where death occurs within about the first six months of life from either liver failure or severe pulmonary dysfunction [1].

Although there is currently no effective treatment for NPC disease, a variety of agents has been evaluated primarily in animal or in vitro models, with 2-hydroxypropyl-ß-cyclodextrin (2HPßCD) showing the best efficacy thus far [19–24]. In a particularly definitive study it was demonstrated that continuous infusion of 2HPßCD into the ventricular system of *Npc1*$^{-/-}$ animals between 3 and 7 weeks of age normalized the biochemical abnormalities and completely prevented the expected neurodegeneration [25]. Earlier studies established that weekly subcutaneous administration of 2HPßCD to *Npc1*$^{-/-}$ mice, starting at 7 days of age, nearly normalized hepatic and whole animal cholesterol pools and prevented development of liver disease [26]. While there was also a

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; bw, body weight; E/L, endosomal/lysosomal; 2HPßCD, 2-hydroxypropyl-ß-cyclodextrin; Npc1, Niemann–Pick C1; Npc2, Niemann–Pick C2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol; UC, unesterified cholesterol

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slowing of cerebellar neurodegeneration and an increase in lifespan, systemic 2HPßCD treatment had little to no effect on the development of progressive pulmonary disease [26]. Subsequent studies further demonstrated lack of impact of systemically administered 2HPßCD on a number of parameters of lung function and pathology in the *Npc1*$^{-/-}$ mouse [27]. There is nothing intrinsically different about UC sequestration in the lungs in NPC1 or NPC2 deficiency because NPC2 replacement therapy in a mouse model of NPC2 disease resulted in a striking reduction in the cholesterol content of several organs including the lungs [28]. Rather, the inaction of 2HPßCD in the NPC lung may simply reflect lack of penetrance at a cellular level. Irrespective of whether this is the case, those studies revealed the paucity of published information about how NPC1 or NPC2 deficiency impacts lung cholesterol metabolism, particularly in early stage disease. In one of our initial studies in the *Npc*^{nih} mouse model we found that even in 1-day old *Npc1*$^{-/-}$ pups the cholesterol concentration in most organs, including the lungs, was elevated [29]. More recently, a murine model of infantile NPC1 deficiency has been described but lungs were not included in the tissues examined [30].

The present studies represent the first systematic evaluation, in quantitative terms, of the ontogenic changes in lung mass, lipid composition, rates of cholesterol and fatty acid synthesis, and also histology in mice with NPC1 or NPC2 deficiency.

2. Materials and methods

2.1. Animals and diets

Control (*Npc1*$^{+/+}$ and *Npc2*$^{+/+}$) and mutant (*Npc1*$^{-/-}$ and *Npc2*$^{-/-}$) mice were generated from respective heterozygous breeding stock on a pure BALB/c background. The NPC1 mice (*Npc1*^{nih}) originated from a colony at the National Institutes of Health (Dr. Peter Pentchev), while heterozygous *Npc2* breeding stock was kindly provided by Dr. Peter Lobel. Depending on the age at which they were to be studied, the mice were genotyped anywhere from 6 to 19 days of age. Unless studied beforehand, all mice were weaned at 19 to 21 days, and thereafter were fed ad libitum a cereal-based, low-cholesterol (0.02% cholesterol, 4% total fat, w/w) diet (No. 7001; Harland Teklad, Madison, WI). They were group-housed in plastic colony cages in rooms with alternating 12-h periods of dark and light and were studied in the fed state at the end of the dark phase. Unless otherwise stated there were comparable numbers of males and females in each group. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Southwestern Medical Center.

2.2. Changes in lung weight as a function of age and Npc1 genotype

In the course of multiple projects with the *Npc1* mouse model we recorded lung weights in a total of 188 untreated animals (94 *Npc1*$^{+/+}$ and 94 *Npc1*$^{-/-}$) over the age range of 8 to 80 days. The lungs were carefully excised, rinsed in physiological saline, blotted and weighed. In some of the mice studied in the age range of 57–60 days, the weight of each lobe (one on the left, and the diaphragmatic, apical, azygous, and cardiac on the right) was recorded.

2.3. Measurement of lung total cholesterol content and proportion of cholesterol present in esterified and unesterified form

Lung total cholesterol content was measured in *Npc1*$^{-/-}$ mice and their matching *Npc1*$^{+/+}$ controls at 8, 19, 29, 35, 49, 57, 63, and 70 days of age. For the *Npc2* mice such measurements were made at 70 days only. The total cholesterol concentration in the lungs (mg/g wet tissue weight) was measured by gas chromatography using stigmastanol as an internal standard [31]. The cholesterol concentration was multiplied by the respective lung weight, to give whole lung cholesterol content (mg/organ). In one study lungs were harvested specifically for measuring the proportion of the total cholesterol present in esterified and unesterified forms. Lungs from *Npc1* mice (57–63 days old) and *Npc2* mice (70–80 days old) were weighed and extracted in chloroform: methanol (2:1 v/v) containing radiolabeled esterified and unesterified cholesterol as internal standards. The esterified and unesterified cholesterol fractions in these extracts were separated by column chromatography and quantitated as described [32].

2.4. Measurement of rates of cholesterol and fatty acid synthesis lungs in vivo

The details of the methods for these measurements are given elsewhere [31]. The ages of the *Npc1* mice used in this study were 8, 23, 52, and 70 days. The mice were given an i.p. injection of [^{3}H]water (~2 mCi/g body wt), and after 1 h, were anesthetized and exsanguinated. The lungs were removed, rinsed and weighed. They were then saponified and their content of radiolabeled digitonin-precipitable sterols (DPS) was measured [31]. The rate of cholesterol synthesis was expressed as the nmol of [^{3}H]water incorporated into DPS per h per whole organ. After the labeled sterols were extracted, the saponified samples were acidified and extracted with hexane. Aliquots of the organic phase were taken for measurement of [^{3}H]-fatty acid content. Rates of fatty acid synthesis were expressed in the same units as for sterol synthesis. For the *Npc2* mice, the rates of lung sterol and fatty acid synthesis were measured at 70 days only.

2.5. Measurement of lung phospholipid and triacylglycerol content

These measurements were made in *Npc1*$^{-/-}$ and matching *Npc1*$^{+/+}$ mice at 8, 25, 50, and 70 days of age. They were not the same animals as those used for the determination of either the lung cholesterol content, or the lung cholesterol and fatty acid synthesis rates. After excision, the lungs were rinsed, weighed and added to chloroform:methanol (2:1 v/v). Procedural losses were corrected for by internal standards. These were either [^{14}C]triolein (American Radiolabeled Chemicals, Inc., St. Louis, MO), or L-α-dipalmitoyl,[choline-methyl-^{3}H]-phosphatidylcholine (Perkin Elmer NEN Radiochemicals, Waltham, MA). An aliquot of this extract was then dried, dissolved in hexane:tert-butyl methyl ether (100:1.5 v/v) and run over a Sep-Pak RC cartridge (500 mg) (Waters Corp., Milford, MA) as described elsewhere [33]. After elution of the cholesteryl esters, the solvent was changed to hexane:tert-butyl methyl ether (96:4 v/v) for elution of triacylglycerols. Finally, the eluting solvent was changed to tert-butyl methyl ether:methanol:ammonium acetate (pH 8.6) (5:8:2 v/v/v) to obtain the phospholipid fraction. This fraction, as well as the one containing the triacylglycerols, was dried and redissolved in 10 ml of chloroform:methanol (2:1 v/v). Aliquots of these solutions were used for quantitation of total triacylglycerol content using Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, Inc., Middletown, VA) [34], or of total phospholipid content by a colorimetric assay using malachite green as described elsewhere [35]. In this method all the phospholipids are digested using perchloric acid at high temperature to liberate phosphorous on a mole per mole basis. It provides a measure of total phospholipid content and not of the proportion of phospholipid species that are present. This requires the use of other techniques including LC/MS/MS as described below. The lung triacylglycerol data were expressed as mg/organ while those for phospholipid were calculated as μmol/organ.

2.6. Determination of lung and liver phospholipid composition

The whole lungs and aliquots of livers from three *Npc1*$^{-/-}$ mice and three matching *Npc1*$^{+/+}$ controls at 49 to 53 days of age were weighed and extracted in choloroform:methanol (2:1 v/v). These pooled extracts were dried under nitrogen and shipped on dry ice to Avanti Polar Lipids, Inc. (Alabaster, AL) where the phospholipid composition was determined by LC/MS/MS. The proportion of each phospholipid detected was expressed as a fraction of the total phospholipid content. For all the