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Impact of the loss of caveolin-1 on lung mass and cholesterol metabolism in mice with and without the lysosomal cholesterol transporter, Niemann-Pick type C1

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Abstract

Caveolin-1 (Cav-1) is a major structural protein in caveolae in the plasma membranes of many cell types, particularly endothelial cells and adipocytes. Loss of Cav-1 function has been implicated in multiple diseases affecting the cardiopulmonary and central nervous systems, as well as in specific aspects of sterol and lipid metabolism in the liver and intestine. Lungs contain an exceptionally high level of Cav-1. Parameters of cholesterol metabolism in the lung were measured, initially in Cav-1-deficient mice (*Cav-1*^{-/-}), and subsequently in *Cav-1*^{-/-} mice that also lacked the lysosomal cholesterol transporter Niemann-Pick C1 (*Npc1*) was also absent (*Cav-1*^{-/-}:*Npc1*^{-/-}). In 50-day-old *Cav-1*^{-/-} mice fed a low- or high-cholesterol chow diet, the total cholesterol concentration (mg/g) in the lungs was marginally lower than in the *Cav-1*^{+/+} controls, but due to an expansion in their lung mass exceeding 30%, whole-lung cholesterol content (mg/organ) was moderately elevated. Lung mass (g) in the *Cav-1*^{-/-}:*Npc1*^{-/-} mice (0.356 ± 0.022) markedly exceeded that in their *Cav-1*^{+/+}:*Npc1*^{+/+} controls (0.137 ± 0.009), as well as in their *Cav-1*^{-/-}:*Npc1*^{+/+} (0.191 ± 0.013) and *Cav-1*^{+/+}:*Npc1*^{-/-} (0.213 ± 0.022) littermates. The corresponding lung total cholesterol content (mg/organ) in mice of these genotypes was 6.74 ± 0.17, 0.71 ± 0.05, 0.96 ± 0.05 and 3.12 ± 0.43, respectively, with the extra cholesterol in the *Cav-1*^{-/-}:*Npc1*^{-/-} and *Cav-1*^{+/+}:*Npc1*^{-/-} mice being nearly all unesterified (UC). The exacerbation of the *Npc1* lung phenotype and increase in the UC level in the *Cav-1*^{-/-}:*Npc1*^{-/-} mice imply a regulatory role of Cav-1 in pulmonary cholesterol metabolism when lysosomal sterol transport is disrupted.

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Keywords

cellular cholesterol trafficking; cholesterol feeding; cholesterol synthesis; pulmonary dysfunction; relative organ weight

1. Introduction

Research using other genetically manipulated mouse models has identified several key proteins that are involved in regulating lung cholesterol homeostasis. These include ATP binding cassette transporter G1 (ABCG1), ATP binding cassette transporter A1 (ABCA1), and sterol-27 hydroxylase (CYP27A1) [1-4]. The loss of any of these proteins results in significant changes in lung cholesterol concentration, particularly in older ABCG1-deficient mice where the level of both unesterified and esterified cholesterol rises appreciably [3]. In ABCA1-deficient mice, lung cholesterol concentration rises modestly, reflecting an increase mainly in the esterified fraction [2, 4], whereas in mice lacking CYP27A1 lung total cholesterol concentration declines marginally [1]. Other proteins are involved in cholesterol trafficking in all tissues including the lungs. For example, within the lysosomal compartment of every cell, three proteins, lipoprotein acid lipase (LAL), Niemann-Pick C1 (Npc1) and Npc2, play a critical role in the processing of cholesterol contained in lipoproteins taken up largely via receptor-mediated endocytosis [5, 6]. In LAL deficiency there is a pronounced increase in tissue cholesteryl ester content whereas loss of function of either Npc1 or Npc2 results in a continuing and highly detrimental expansion of tissue unesterified cholesterol content in all organs. LAL, Npc1 and Npc2 deficiency each result in a distinctive lung phenotype [7-11].

Another protein that could conceivably play a role in regulating cholesterol homeostasis in the lungs is Caveolin-1 (Cav-1), a structural protein in caveolae within plasma membranes that is highly expressed in pulmonary tissue principally in endothelial cells and type I pneumocytes [12, 13]. Caveolae are critically important in facilitating various processes including endocytosis, transcytosis, and signal transduction [14, 15]. Investigations using Cav-1 knockout mice were first reported in 2001. Initially, four of these models were independently generated, all using a mixture of different mouse strains [16-19]. Since then, Cav-1-deficient mice on a single background, either C57BL/6, FVB or BALB/c, have been developed [20-22]. There is a plethora of published data describing the impact that abnormalities in Cav-1 expression have on several major organ systems. This includes multiple changes to cardiopulmonary pathology and function [17, 18, 23-25], neurologic disorders [26, 27], defective insulin-regulated lipogenesis and lipid droplet formation [22, 28-31], and decreased intestinal fat absorption [32]. Lung enlargement is caused by hypercellularity and a thickening of the alveolar septa [13, 16, 33]. Importantly, re-expression of Cav-1 in endothelium reverses the vascular, cardiac, and pulmonary defects evident in *Cav-1*^{-/-} mice [34]. While loss of Cav-1 is mostly detrimental in some organ systems, in a mouse atherosclerosis model, the elimination of Cav-1 reduced disease severity [35, 36]. Another positive impact is protection from diet-induced obesity [37].

Other publications have focused on the effects of Cav-1 deficiency on particular aspects of hepatic and intestinal cholesterol metabolism as well as plasma lipoprotein composition [20, 31, 38-40]. One study found that in *Cav-1*^{-/-} mice there was a marginal rise in hepatic cholesterol levels, a reduction in very low density lipoprotein secretion, and an increase in HDL levels with a greater enrichment of esterified cholesterol in the HDL [20]. More recently, detailed studies using multiple approaches including three different *Cav-1*^{-/-} mouse models revealed a role of Cav-1 in bile acid signaling, synthesis, and trafficking [31]. The hypothesis being tested here is whether the types of regulatory influences that Cav-1 is now thought to exert on specific aspects of sterol metabolism within the liver are unique to that organ, or whether this protein plays a role in regulating cholesterol homeostasis in multiple tissues. Of particular interest are the lungs which manifest Cav-1 protein expression levels far exceeding those in the liver. Despite the extensive literature detailing the lung phenotype in Cav-1-deficient mice, there are no published data on any aspect of cholesterol metabolism in this model. In the present studies we used mice lacking Cav-1 only, or both Cav-1 and Npc1. Our rationale in using this particular double knockout, in addition to mice deficient in Cav-1 only, was that the loss of Npc1 alone results in a marked lung phenotype characterized in part by decisive changes in cholesterol metabolism resulting from defective lysosomal sterol transport. Given the role of caveolae in cellular cholesterol movement, and the disruption of intracellular sterol transport caused by deficiency of Npc1, it seemed a reasonable premise that in lung tissue deficient in both the main structural protein of caveolae and Npc1, there might be more overt changes in cholesterol metabolism than seen with either Cav-1 or Npc1 deficiency alone. The data show that while Cav-1 deficiency alone did not alter cholesterol metabolism in the lungs, it exacerbated the Npc1 lung phenotype with an accompanying expansion of the tissue level of unesterified cholesterol.

2. Materials and methods

2.1. Animals and diets

Male Cav-1 deficient (*Cav-1*^{-/-}) mice [17], on a pure FVB background [21], were bred to wild-type (*Cav-1*^{+/+}) females (also FVB) to establish a colony of *Cav-1*^{+/-} breeding stock. These were used to generate all the *Cav-1*^{-/-} and matching *Cav-1*^{+/+} control mice used in these studies. Depending on the experiment, these mice were studied at 24, 50, 100 or 195 days of age. To generate *Cav-1*^{-/-}:*Npc1*^{-/-} mice, we first crossed *Cav-1*^{-/-} males (FVB) with *Npc1*^{+/+} females (BALB/c). Offspring that were heterozygous for both Cav-1 and Npc1 (*Cav-1*^{+/-}:*Npc1*^{+/-}) were in turn used to produce mice of the four genotypes needed for study (*Cav-1*^{+/+}:*Npc1*^{+/+}, *Cav-1*^{-/-}:*Npc1*^{+/+}, *Cav-1*^{+/+}:*Npc1*^{-/-}, and *Cav-1*^{-/-}:*Npc1*^{-/-}). From a large number of litters we obtained just 5 double-knockouts, and 4 to 8 siblings of each of the other three genotypes needed for comparison to the *Cav-1*^{-/-}:*Npc1*^{-/-} pups. All litters were weaned at 21 days onto a cereal-based rodent chow diet (Teklad 7001 Madison, WI). This formulation had an inherent cholesterol and crude fat content of 0.02 and 4% (wt/wt), respectively. In one study involving cholesterol-fed mice, the level of cholesterol in the diet was raised to 0.5% (wt/wt). All mice were group-housed in rooms with alternating 12-h periods of dark and light, and were studied in the fed state toward the end of the dark phase. Pups were genotyped at weaning by automated real-time quantitative PCR (Transnetix, Inc., Cordova, TN). All experiments were approved by the

Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

2.2. Rate of cholesterol synthesis

Cholesterol synthesis in the lungs was measured in vivo using [^3H]water as described [41]. One hour after the mice were given a bolus of [^3H]water intraperitoneally (~40 mCi), the lungs were removed, rinsed, blotted and weighed. They were not lavaged before excision. The lungs were saponified in alcoholic KOH after which the radiolabeled sterols were extracted and quantitated [41]. The rate of cholesterol synthesis was calculated as the nanomoles of [^3H]water incorporated into sterols per hour per gram wet weight of tissue (nmol/h/g). Rates per whole-lung were obtained by multiplying the rate per gram by the organ weight.

2.3. Concentration of total, unesterified and esterified cholesterol in lung tissue

The total cholesterol concentration was quantified using gas chromatography with stigmasterol as an internal standard [41]. For the determination of esterified and unesterified cholesterol concentrations, the lungs were extracted in chloroform/methanol (2:1 v/v) in the presence of two internal standards, [4- ^{14}C]cholesteryl oleate and [1.2 $^3\text{H}(\text{N})$]cholesterol [42]. The esterified and unesterified cholesterol fractions were separated using a silica column (Sep-Pak Vac RC 500 mg; Waters, Milford MA). The eluted sterols were dried and saponified in alcoholic KOH and extracted into petroleum ether. The cholesterol mass was determined by gas chromatography and corrected for recovery of the radiolabeled standards. These data were calculated as mg per gram wet weight of tissue (mg/g). Whole-lung cholesterol contents were determined by multiplying the concentration values by the lung weights.

2.4. Relative mRNA expression analysis

Lung aliquots were promptly frozen in liquid nitrogen and RNA was extracted using the RNA Stat-60 reagent (Tel-Test Inc. Friendswood, TX). Relative levels of mRNA expression were measured using a quantitative real-time PCR assay as previously described [43]. All analyses were determined by the comparative cycle number at threshold method [44], with cyclophilin as the internal control. A prior evaluation of multiple housekeeping genes showed cyclophilin to be superior because its expression level in lung across all genotypes was the most constant. Relative mRNA levels in individual animals were determined by expressing the amount of mRNA found relative to that obtained for the wildtype controls (*Cav-1*^{+/+}:*Npc1*^{+/+}), which in each case was arbitrarily set at 1.0. The primer sequences used to measure mRNA levels are given in Table 1.

2.5. Analysis of data

Values are mean $\pm$ 1 SEM for the specified number of animals. GraphPad Prism 6 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. Depending on the design of each experiment, differences between mean values were tested for statistical significance ($p < 0.05$) by an unpaired two-tailed Student's *t*-test, a one-way analysis of

variance (ANOVA) with genotype as the variable, or a two-way ANOVA with either genotype and diet, or genotype and age as variables.

3. Results

3.1. Marked increase in lung mass in *Cav-1^{-/-}* mice at 24, 50, and 100 days of age was not accompanied by any change in total cholesterol concentration

These measurements were made in female mice only, except for the 24-day-old animals which included some males of both *Cav-1* genotypes. The genotypic and ontogenic changes in lung weight, as well as cholesterol concentration and content, are illustrated in Fig. 1. In keeping with the known phenotype of *Cav-1^{-/-}* mice, a marginally lower body weight was evident (data not shown) and this was a factor in making their relative lung weights higher than those in the *Cav-1^{+/+}* controls. Even at 24 days of age, the *Cav-1^{-/-}* mice had a significantly greater lung mass (Fig. 1A). The magnitude of the genotypic difference in lung weight at both 50 and 100 days was the same as that evident at 24 days. The genotype-related differences in relative lung weight (Fig. 1B) mirrored those seen in the absolute weight. For both the *Cav-1^{-/-}* and *Cav-1^{+/+}* mice at 24 days, relative lung weights were clearly greater than they were in the older mice. In contrast to the clear effects of *Cav-1* deficiency on lung mass, there were no corresponding genotypic differences in lung cholesterol concentration although there was a trend toward lower values in the *Cav-1^{-/-}* mice (Fig. 1C). Thus the marginally higher lung cholesterol contents in the *Cav-1^{-/-}* mice at all three ages (Fig. 1D) were due entirely to the larger lung mass in each case.

Although the data are not shown, additional measurements of lung mass and cholesterol content were made in six 195-day-old *Cav-1^{-/-}* males and their five *Cav-1^{+/+}* littermates maintained on the rodent chow diet since weaning. Lung mass (g) in the *Cav-1^{-/-}* mice averaged 0.232 ± 0.008 g vs 0.166 ± 0.005 in their *Cav-1^{+/+}* controls. The corresponding whole-lung cholesterol contents (mg/organ) in these groups were 1.086 ± 0.039 vs 0.919 ± 0.021 , respectively.

3.2. Cholesterol synthesis in the lungs was unchanged by caveolin-1 deficiency

The rate of cholesterol synthesis in any organ, particularly if measured *in vivo*, is unquestionably one of the best barometers for gauging how a genetic manipulation impacts cholesterol handling in that organ. Such a study was therefore carried out in female *Cav-1^{-/-}* mice and their *Cav-1^{+/+}* controls at 50 and 100 days of age. As shown by the data in Table 2, the synthesis rate, expressed per gram of lung, was modestly lower in the *Cav-1^{-/-}* mice, but when expressed on a whole-lung basis, no genotypic difference in synthesis rate was evident.

3.3. Impact of feeding a high cholesterol diet on lung weights and tissue cholesterol concentration in the *Cav-1^{-/-}* mice was not different from that in their *Cav-1^{+/+}* controls

The next study, carried out in male mice, addressed the question of whether a marked and sustained elevation in dietary cholesterol intake starting soon after weaning might reveal an impact of *Cav-1* deficiency on cholesterol handling by the lungs that was not detectable in mice fed a low-cholesterol basal chow diet. Here we took account of an earlier finding that,

although Cav-1 protein has been shown to be highly expressed in the small intestine of mice [12], the loss of Cav-1 does not affect cholesterol absorption in this species [39]. The cholesterol-enriched diet we used increased the cholesterol intake of the mice about 25-fold. Only marginal rises in the plasma total cholesterol concentration after 3 wks on the high-cholesterol diet were evident (Fig. 2A). In addition, no genotypic differences in plasma cholesterol levels were detected in keeping with the data of Razani et al [37]. No diet-related differences were detected for mice of either genotype in relative lung weight (Fig. 2B), or in lung cholesterol concentration (Fig. 2C) and content (Fig. 2D).

3.4. Cav-1^{-/-}:Npc1^{-/-} mice exhibited markedly greater lung weights, and total cholesterol concentrations and contents compared to their Cav-1^{-/-}:Npc1^{+/+} and Cav-1^{+/+}:Npc1^{-/-} littermates

The average body weight of the mice deficient in both Cav-1 and Npc1 at 50 days of age was the same as that of their littermates lacking only Npc1 (Fig. 3A). There was a trend for the body weight of these two groups to be less than that of the mice deficient in only Cav-1, which in turn had a lower mean body weight than the *Cav-1^{+/+}:Npc1^{+/+}* controls ($p > 0.05$). There were pronounced genotypic differences in absolute lung weights, particularly for the *Cav-1^{-/-}:Npc1^{-/-}* mice (Fig. 3B). In these mice, the increase in lung mass exceeded the combined increases associated with deficiency of either Cav-1 or Npc1 alone. The lung total cholesterol concentration (Fig. 3C) and content (Fig. 3D) data reveal striking genotypic differences. The 2.8-fold greater cholesterol concentration in the lungs of *Cav-1^{+/+}:Npc1^{-/-}* is in keeping with the known lung phenotype of Npc1-deficient mice [11]. Unexpectedly, the loss of Cav-1 function in the face of Npc1 deficiency led to a significantly higher total cholesterol concentration in the *Cav-1^{-/-}:Npc1^{-/-}* mice compared to that in their littermates deficient in just Npc1 (Fig. 3C). This effect is further magnified when the lung cholesterol data are expressed on a whole-organ basis (Fig. 3D).

3.5. Additional cholesterol present in lungs of Cav-1^{+/+}:Npc1^{-/-} and Cav-1^{-/-}:Npc1^{-/-} mice was essentially all unesterified

The data described in Fig. 3C and Fig. 3D prompted the measurement of the absolute concentrations of unesterified (UC) and esterified cholesterol (EC) in the lungs of mice of all four genotypes (Table 3). These data show that, irrespective of genotype, the proportion of total cholesterol contained in the esterified fraction was less than 5%, particularly in the *Cav-1^{+/+}:Npc1^{-/-}* and *Cav-1^{-/-}:Npc1^{-/-}* groups. The more significant finding here was that, while the build up of cholesterol in the *Cav-1^{+/+}:Npc1^{-/-}* mice and the double knockouts was nearly all in the UC fraction, the increment in UC in the latter group was significantly greater than in the mice deficient in Npc1 only. The levels of UC and EC in organs other than the lungs of these mice were not determined.

3.6. Degree of increase in mRNA expression levels for genes involved in surfactant production, inflammation, and apoptosis in the lungs of Cav-1^{-/-}:Npc1^{-/-} mice was not greater than that in Cav-1^{+/+}:Npc1^{-/-} mice

The relative mRNA data in Fig. 4 reveal three main findings. First, in no case was the mRNA expression level in the *Cav-1^{-/-}:Npc1^{+/+}* mice different than that for the

Cav-1^{+/+}:*Npc1*^{+/+} controls. Second, for six of the twelve genes (Fig 4A, 4B, 4C, 4F, 4G, and 4I), the mRNA level in the *Cav-1*^{+/+}:*Npc1*^{-/-} mice was significantly greater than the level for the *Cav-1*^{+/+}:*Npc1*^{+/+} mice. Third, with one exception (Fig 4C), the degree of change in the mRNA level for all genes in the mice deficient in both Cav-1 and Npc1 was no different than in the mice lacking just Npc1. The mRNA level for LDLR (Fig 4C) in the *Cav-1*^{-/-}:*Npc1*^{-/-} mice was not as elevated as was the case in the littermates lacking only Npc1.

4. Discussion

The main conclusion drawn from the initial studies with the *Cav-1*^{-/-} mice was that, depending on how the data were normalized, cholesterol metabolism in their lungs showed little to no change in the face of a marked increase in tissue mass which was equally manifest in the 24-day old *Cav-1*^{-/-} mice as it was in their counterparts at 50 and 100 days of age. It should be noted here that while there was a clear genotypic effect on lung mass at all three ages, lung weights, within genotypes, changed only marginally after 24 days of age. At least for the *Cav-1*^{+/+} mice, this was not unexpected because lung growth normally occurs mostly in the early stages of development. This was illustrated in detailed studies in the mouse by Amy et al. [45] which revealed that by about 24 days after birth, the changes in lung weight, especially relative to body weight, are far less than they are in the first ten days after birth. The data in Fig. 2 showed that prolonged feeding of a high cholesterol diet, starting just after weaning, did not have any additional impact on lung mass or cholesterol levels in the *Cav-1*^{-/-} mice. As discussed elsewhere, the increase in lung weight associated with Cav-1 deficiency reflects thickening of the alveolar wall, hypercellularity, and changes in pulmonary vasculature [23, 24].

The two most striking features of the double-knockouts compared to siblings deficient in just Cav-1 or Npc1 were the markedly greater lung mass, and the expansion of lung UC content to levels significantly above those arising from Npc1 deficiency alone. This higher UC content in the double knockouts reflected not just a greater lung mass but also more UC per gram of tissue. These findings prompt several questions. The first one relates to the intracellular distribution of the extra UC in the lungs of the double knockouts vs those of the *Cav-1*^{+/+}:*Npc1*^{-/-} mice. On a mg/g basis, there was about 33% more UC in the lung tissue of the *Cav-1*^{-/-}:*Npc1*^{-/-} mice (Table 2). Most, if not all of this extra UC is presumably localized to a particular subcellular site such as in the late endosomal/lysosomal compartment because elsewhere in the cell it could potentially exert cytotoxic effects through a variety of mechanisms [46], assuming that 33% more UC would be sufficient to elicit such a response. Several aspects of the mRNA expression data in Fig. 4 lend support to the conclusion that this UC is compartmentalized. If this were not the case then the rate of cholesterol synthesis in the lungs of the double knockouts vs that in the *Cav-1*^{+/+}:*Npc1*^{-/-} mice would likely reflect some inhibition. The mRNA data for both HMGCR (Fig. 4A) and HMGCS (Fig. 4B), surrogate measures of the rate of sterol synthesis, suggest that the rate was comparable in these two groups. Likewise, one might anticipate higher levels of expression of mRNA for the apoptosis markers, CASP3 (Fig. 4K) and CASP8 (Fig. 4L). There was no indication that this was so. Consistent with this finding was the observation that the mRNA levels for markers of macrophage presence and inflammation were not

different in the lungs of the mice deficient in both Npc1 and Cav-1 vs those lacking only Npc1 (Fig. 4E - Fig. 4H).

The second question, like those that follow, will require additional analyses and experiments that are beyond the scope of the present studies. It has to do with the types of cells where the UC is accumulating, and if this is the same in the double knockouts as it is in the mice deficient in only Npc1. Recent work by Roszell et al. using immunocytochemistry showed that cholesterol accumulation in the lungs of Npc1-deficient mice is confined to the lamellar bodies of Type II pneumocytes [10] which is where surfactant is produced [47]. It is noteworthy that the lung mRNA data in Fig. 4 show that for surfactant proteins (SP) A and D there was no change in the mice deficient in only Cav-1. In contrast, there was a clear increase in mRNA for SP-A in the mice lacking only Npc1, with a comparable change seen for their *Cav-1^{-/-}:Npc1^{-/-}* counterparts (Fig. 4I).

A third question is whether the higher concentration (mg/g) of UC in the lungs of the double knockouts represents an increase in cholesterol entry into the lung sterol pool, or/and a slower turnover rate. Technically, this will be a difficult question to address partly because the difference in UC concentration was not dramatic. The relative mRNA expression level for the LDL receptor (Fig. 4C), while not necessarily reflective of LDLR protein and activity levels, implies that LDL might not be a source of the extra cholesterol. Although this cholesterol was almost entirely in the UC fraction, there was also a marginal but significant rise in the amount of EC in the lungs of the *Cav-1^{-/-}:Npc1^{-/-}* mice compared to the littermates lacking only Npc1. This could be taken as an indication of an increased presence of macrophages in the lungs of the double knockouts vs the mice deficient in only Npc1. However, the mRNA data for SOAT1 (Fig. 4E) and CD68 (Fig. 4G) together suggest this was probably not the case.

Two other interrelated questions warranting investigation are whether the lung UC concentration in the double knockout would continually increase with age more than in *Cav-1^{+/+}:Npc1^{-/-}* mice, and if so, what impact this might have on their longevity. Pulmonary dysfunction in Npc1 and Npc2 deficiency can itself be a cause of premature death [48]. Depending on a number of factors, including their strain background and the type of mutation in the Npc1 gene, the lifespan of *Npc1^{-/-}* mice is usually around 80 to 85 days [11, 49]. It is difficult to predict how the loss of Cav-1 function in the Npc1 model might alter its lifespan given that for *Cav-1^{-/-}* mice one study found a 50% reduction in lifespan, whereas another laboratory reported no detectable change [26, 50]. Ideally, such a longevity study would need to be conducted in mice of a single strain background.

Taken together, the present data show for the first time that the loss of Cav-1 function, at least in a mouse model with defective lysosomal transport of unesterified cholesterol, leads to an alteration in steady-state levels of cholesterol in lung tissue. Although the mRNA measurements were interpreted to mean that this additional UC in the *Cav-1^{-/-}:Npc1^{-/-}* mice was possibly contained in the pool of sterol already sequestered in the late endosomal/lysosomal compartment due to the absence of Npc1 alone, other approaches will be needed to explore the mechanisms for the exacerbation of the lung phenotype in the *Cav-1^{-/-}:Npc1^{-/-}* mice. A combination of electron microscopy, histology, and

immunocytochemistry as applied elsewhere to lungs of various models with Npc1 or Npc2 deficiency may be fruitful [10]. Another approach would be the measurement of lung UC and EC content and related parameters of cholesterol metabolism in Cav-1-deficient mice also lacking other proteins such as ABCA1, or LAL in particular.

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Abbreviations

ABCA1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
bw	body weight
Cav-1	Caveolin-1
EC	esterified cholesterol
CYP27A1	sterol-27 hydroxylase
FVB	Friend Leukemia Virus
LAL	lysosomal acid lipase
Npc1	Niemann-Pick C1
Npc2	Niemann-Pick C2
UC	unesterified cholesterol

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Highlights

- Young adult Caveolin-1 KO mice exhibited reduced body weight and expanded lung mass
- The higher lung weights in the Cav-1 KO mice were evident by the time of weaning
- Lung cholesterol synthesis and cholesterol levels were unchanged in Cav-1 KO mice
- A high cholesterol diet did not exacerbate the lung phenotype in the Cav-1 KO mice
- Lungs in Cav-1:NPC1 DKO mice have increased lung mass and cholesterol content

Fig. 1. Absolute and relative lung weight, and lung cholesterol concentration and content, each as a function of age, in *Cav-1*^{-/-} and *Cav-1*^{+/+} mice. Absolute lung weight (A) was expressed relative to body weight (B). Lung total cholesterol concentration (C) was multiplied by absolute lung weight to determine lung cholesterol content (D). The 100-day-old mice were all females, like those at 50 days. At 24 days, there were female and male mice of each *Cav-1* genotype. Values represent the mean ± 1 SEM of data from 3-7 mice in each group.

Different letters (a-d) denote statistically different values ($p < 0.05$) as determined by 2-way ANOVA with age and genotype as variables.

Fig. 2.

Effect of feeding a high-cholesterol diet on lung weight and cholesterol content in *Cav-1*^{-/-} and *Cav-1*^{+/+} mice at 50 days of age. Male *Cav-1*^{-/-} and *Cav-1*^{+/+} mice were fed either the basal diet alone or the same diet with added cholesterol (0.5% wt/wt) for 3 weeks starting at 28 or 29 days of age. Plasma total cholesterol concentration (A), relative lung weight (B) and lung total cholesterol concentration (C) and content (D) were measured. Values represent the mean $\pm$ 1 SEM of data from 5-7 mice in each group. Different letters (a-c)

denote statistically different values ($p < 0.05$) as determined by 2-way ANOVA with genotype and dietary cholesterol level as variables.

Fig. 3.

Body and lung weights, and lung cholesterol concentration and content in mice deficient in either Caveolin-1 or Niemann-Pick C1, or both of these proteins. These mice were generated as described in Materials and Methods, and were fed a basal rodent chow diet from weaning until studied at 50 days of age. The number of mice for each genotype was 4 *Cav-1*^{+/+}:*Npc1*^{+/+}, 8 *Cav-1*^{-/-}:*Npc1*^{+/+}, 6 *Cav-1*^{+/+}:*Npc1*^{-/-} and 5 *Cav-1*^{-/-}:*Npc1*^{-/-}, with approximately equal numbers of males and females in each group. Values are the mean $\pm$ 1 SEM of data from the specified number of animals. Different letters (a-c) denote

statistically different values ($p < 0.05$) as determined by 1-way ANOVA with genotype as the variable.

Fig. 4. Relative mRNA expression levels in the lungs of mice deficient in either Caveolin-1, Niemann-Pick C1, or both of these proteins. These mice were generated as described in Materials and Methods and were fed a basal chow diet until studied at 50 days of age. The lungs were used for the mRNA analyses shown here, as well as for measurement of the tissue content of unesterified and esterified cholesterol (Table 3). The gene names and primer sequences are given in Table 1. The amount of mRNA found was expressed relative to that obtained for the *Cav-1*^{+/+}:*Npc1*^{+/+} mice, which in each case was arbitrarily set at 1.0. Values are the mean $\pm$ 1 SEM of data from the same numbers of mice for each genotype as given in Fig. 3 and Table 3. Different letters (a-c) denote statistically different values ($p < 0.05$) as determined by 1-way ANOVA with genotype as the variable.

Table 1

Primer sequences used for analyzing mouse mRNA expression in lung

Gene	Gene name	Accession#	Primer sequence (5'-3')
<i>Cyclophilin</i>	Cyclophilin	M60456	F: TGGAGAGCACCAAGACAGACA R: TGCCGGAGTCGACAATGAT
<i>Abcg1</i>	ATP-binding cassette G1	NM_009593	F: GCTGTGCGTTTTGTGCTGTT R: TGCAGCTCCAATCAGTAGTCCTAA
<i>Soat1(Acat1)</i>	Sterol O-acyltransferase 1	NM_009230	F: AGTATGCCCTCGCCATCTG R: CCGACTGTCTGTTAACAATGAAGT
<i>CD11C</i>	CD11c antigen; Integrin alpha X	NM_021334	F: CTTCAATTCTGAAGGGCAACCT R: CACTCAGGAGCAACACCTTTT
<i>CD68</i>	CD68 antigen	NM_009853	F: CCTCCACCCTCGCCTAGTC R: TTGGGTATAGGATTCGGATTGA
<i>TNFA</i>	Tumor necrosis factor alpha	NM_013693	F: CTGAGGTCAATCTGCCCAAGTAC R: CTTACAGAGCAATGACTCCAAAG
<i>Hmgcr</i>	Hydroxymethylglutaryl coenzyme A reductase	NM_008255	F: CTTGTGGAATGCCTTGTGATTG R: AGCCGAAGCAGCACATGAT
<i>Hmgcs</i>	Hydroxymethylglutaryl coenzyme A synthase	NM_145942	F: GCCGTGAACTGGGTCGAA R: GCATATATAGCAATGTCTCCTGCAA
<i>Ldlr</i>	Low density lipoprotein receptor	NM_010700	F: GAGGAACTGGCGGCTGAA R: GTGCTGGATGGGGAGGTCT
<i>SPA(Sfpal)</i>	Surfactant associated protein A1	NM_023134	F: TCCAGGGTTTCCAGCTTACCT R: GACAGCATGGATCCTTGCAAG
<i>SPD(Sfpd)</i>	Surfactant associated protein D	NM_009160	F: GGAATCAAGGGGACAGAG R: AGCTTTCTGATAGTGGGAGAAGG
<i>Casp3</i>	Caspase 3	NM_009810	F: CATAAGAGCACTGGAATGTCATCTC R: CCCATGAATGTCTCTCTGAGGTT
<i>Casp8</i>	Caspase 8	NM_009812	F: CCTGAGGGAAAGATGTCCTCAA R: GTCGTCTTTATTGCTCACGTCATAG

Table 2

Rate of cholesterol synthesis in the lungs of female *Cav-1*^{+/+} and *Cav-1*^{-/-} mice at 50- and 100-days of age.

Genotype	Age (days)	Number of mice	Cholesterol synthesis	
			(nmol/h/g)	(nmol/h/organ)
<i>Cav-1</i> ^{+/+}	50	6	93.6 ± 4.3	12.2 ± 0.7
<i>Cav-1</i> ^{-/-}	50	9	74.6 ± 4.9*	12.8 ± 0.9
<i>Cav-1</i> ^{+/+}	100	4	117.3 ± 11.5	13.5 ± 1.3
<i>Cav-1</i> ^{-/-}	100	4	81.5 ± 9.4	14.4 ± 1.5

All mice were fed the basal chow diet with no additions. The rate of cholesterol synthesis was measured in vivo as described in Material and Methods. Values are the mean ± 1 SEM of data from the specified number of animals.

* Significantly different compared to value for age-matched *Cav-1*^{+/+} controls ($p < 0.05$).

Table 3

Concentration and content of unesterified and esterified cholesterol in the lungs of mice deficient in either caveolin-1 or Niemann-Pick C1, or both of these proteins

Genotype	Number of mice	Cholesterol concentration in lungs (mg/g)		Cholesterol content in lungs (mg/organ)	
		Unesterified	Esterified	Unesterified	Esterified
<i>Cav-1^{+/-};Npc1^{+/-}</i>	4	4.98 ± 0.07 ^a	0.196 ± 0.035 ^a	0.68 ± 0.04 ^a	0.027 ± 0.006 ^a
<i>Cav-1^{-/-};Npc1^{+/-}</i>	8	4.87 ± 0.17 ^a	0.202 ± 0.019 ^a	0.92 ± 0.04 ^a	0.039 ± 0.006 ^a
<i>Cav-1^{+/-};Npc1^{-/-}</i>	6	14.21 ± 0.82 ^b	0.218 ± 0.012 ^a	3.07 ± 0.42 ^b	0.047 ± 0.006 ^a
<i>Cav-1^{-/-};Npc1^{-/-}</i>	5	18.85 ± 1.07 ^c	0.307 ± 0.029 ^b	6.63 ± 0.16 ^c	0.111 ± 0.017 ^b

Values are the mean ± 1 SEM of data from the specified number of animals, with each group containing approximately equal numbers of males and females. Within the values for unesterified cholesterol, or those for esterified cholesterol, different letters (a-c) denote statistically different values ($p < 0.05$) as determined by one-way ANOVA with genotype as the variable.