Npc1 haploinsufficiency promotes weight gain and metabolic features associated with insulin resistance

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A recent population-based genome-wide association study has revealed that the Niemann–Pick C1 (NPC1) gene is associated with early-onset and morbid adult obesity. Concurrently, our candidate gene-based mouse growth study performed using the BALB/cJ NPC1 mouse model (Npc1) with decreased Npc1 gene dosage independently supported these results by suggesting an Npc1 gene–diet interaction in relation to early-onset weight gain. To further investigate the Npc1 gene in relation to weight gain and metabolic features associated with insulin resistance, we interbred BALB/cJ Npc1¹/² mice with wild-type C57BL/6J mice, the latter mouse strain commonly used to study aspects of diet-induced obesity and insulin resistance. This breeding produced a hybrid (BALB/cJ–C57BL/6J) Npc1¹/² mouse model with increased susceptibility to weight gain and insulin resistance. The results from our study indicated that these Npc1¹/² mice were susceptible to increased weight gain characterized by increased whole body and abdominal adiposity, adipocyte hypertrophy and hepatic steatosis in the absence of hyperphagia. Moreover, these Npc1¹/² mice developed abnormal metabolic features characterized by impaired fasting glucose, glucose intolerance, hyperinsulinemia, hyperleptinemia and dyslipidemia marked by an increased concentration of cholesterol and triacylglycerol associated with low-density lipoprotein and high-density lipoprotein. The overall results are consistent with a unique Npc1 gene–diet interaction that promotes both weight gain and metabolic features associated with insulin resistance. Therefore, the NPC1 gene now represents a previously unrecognized gene involved in maintaining energy and metabolic homeostasis that will contribute to our understanding concerning the current global epidemic of obesity and type 2 diabetes mellitus.

INTRODUCTION

The Niemann–Pick C1 (NPC1) gene has been primarily investigated in relation to an autosomal–recessive lipid-storage disorder characterized by neonatal jaundice, hepatosplenomegaly, vertical gaze palsy, ataxia, dystonia and progressive neurodegeneration resulting in death most often during the second decade (1,2). Moreover, additional studies have recently demonstrated that NPC1 patients possess decreased high-density lipoprotein (HDL) cholesterol levels that are inversely associated with the biochemical severity of NPC1 mutations and the removal of cellular low-density lipoprotein (LDL)-derived cholesterol mediated by apolipoprotein A-1 (3,4). The NPC1 gene has been localized to chromosome 18 and shown to encode a large and complex multi-spanning transmembrane protein with extensive structural homology with members of the resistance-nodulation-division family of prokaryotic permeases (5,6). With respect to the various structural motifs, the NPC1 protein contains 13 membrane-spanning helices and 3 large luminal domains, among which an N-terminal domain and sterol-sensing domain independently bind cholesterol (7,8). To date, the exact function of

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the NPC1 protein remains controversial, since conflicting results have been reported concerning the lipid substrate (cholesterol, sphingolipid or fatty acid) that this protein transports across the limiting membrane of late endosomes/lysosomes (5,9,10). Nonetheless, studies have determined that expression of the NPC1 gene is regulated through the sterol regulatory element-binding protein pathway, consistent with the NPC1 protein having a central role in maintaining cellular, tissue and whole body lipid homeostasis (11,12).

A recent genome-wide association study has revealed that the NPC1 gene is associated with early-onset and morbid adult obesity in European populations (13). This study was performed using nearly 1400 obese Europeans compared with a similar number of age-matched normal weight controls, and then confirmed with an additional 2100 obese and 2400 normal weight individuals. At the time, it was unknown whether the NPC1 gene non-synonymous single nucleotide polymorphism (SNP) variants (rs1805081 and rs1805082 encoding His215Arg and Ile858Val, respectively) found in this study increased or decreased NPC1 protein function. However, we had concurrently performed a candidate-gene-based mouse growth study using the BALB/cJ NPC1 mouse model (Npc1), which possesses a transposon insertion resulting in a null mutation characterized by a severely truncated NPC1 protein (14–16). The results from these studies demonstrated that when compared with Npc1 homozygous normal (Npc1+/+) mice, Npc1 heterozygous (Npc1+/-) mice with 50% decreased Npc1 gene dosage had significant weight gain when fed a high-fat diet, but not when fed a low-fat diet, consistent with a gene–diet interaction (17).

To further investigate the Npc1 gene in relation to weight gain and metabolic features associated with insulin resistance, we interbred BALB/cJ Npc1+/- mice with wild-type C57BL/6J mice, the latter mouse strain commonly used to study aspects of diet-induced obesity and insulin resistance. This breeding produced a hybrid (BALB/cJ-C57BL/6J) Npc1+/- mouse model with increased susceptibility to weight gain and insulin resistance (18,19). The results from our study indicated that these Npc1+/- mice were susceptible to increased weight gain characterized by increased whole body and abdominal adiposity, adipocyte hypertrophy and hepatic steatosis in the absence of hyperphagia. Moreover, these Npc1+/- mice developed abnormal metabolic features characterized by impaired fasting glucose, glucose intolerance, hyperinsulinaemia, hyperleptinemia and dyslipidemia marked by an increased concentration of cholesterol and triacylglycerol associated with LDL and HDL. The overall results are consistent with a unique Npc1 gene–diet interaction in promoting both weight gain and metabolic features associated with insulin resistance. Therefore, the NPC1 gene now represents a previously unrecognized gene involved in maintaining energy and metabolic homeostasis that will contribute to our understanding concerning the current global epidemic of obesity and type 2 diabetes mellitus.

RESULTS

The Npc1+/- mice fed either a low- or high-fat diet had modest but significantly increased body weights (increased 15.1% from 29.1 ± 0.5 to 33.5 ± 1.0 g and 32.5% from 32.3 ± 0.9 to 42.8 ± 1.4 g, respectively) compared with Npc1+/- mice fed the same diets (Fig. 1A). Moreover, the Npc1+/- mice fed either a low- or high-fat diet had significantly increased liver weights (increased 18.9% from 1.48 ± 0.06 to 1.76 ± 0.08 g and 51.6% from 1.59 ± 0.06 to 2.41 ± 0.14 g, respectively) compared with Npc1+/- mice fed the same diets (Fig. 1B). And finally, Npc1+/- mice fed either a low- or high-fat diet had significantly increased gonadal adipose weights (increased 42.8% from 0.14 ± 0.06 g to 0.83 ± 0.03 g and 72.6% from 1.13 ± 0.09 to 1.94 ± 0.12 g, respectively) compared with Npc1+/- mice fed the same diets (Fig. 1C). Therefore, Npc1 haploinsufficiency was associated with increased body weight, liver weight and gonadal adipose weight when mice were fed either a low- or high-fat diet.

The consumption of nutritional energy for Npc1+/- and Npc1+/- mice fed either a low- or high-fat diet was determined between 30 and 70 days of age. The results indicated no significant difference in the amounts of nutritional energy (kcal/day/g of body weight) consumed for Npc1+/- and Npc1+/- mice when fed the same diets (Fig. 2). However, it was interesting to note that Npc1+/- and Npc1+/- mice fed a high-fat diet consumed less nutritional energy than Npc1+/- and Npc1+/- mice fed a low-fat diet, and that Npc1+/- and Npc1+/- mice consumed less nutritional energy than Npc1+/- and Npc1+/- mice fed the same diets. An asterisk indicates a significant difference (P < 0.05) in weight when comparing Npc1+/- mice with Npc1+/- mice fed the same diet. Values are represented by means ± SEM (n = 15).

Figure 1. Body weights, liver weights and adipose weights. The body weight (A), liver weight (B) and gonadal adipose weight (C) for Npc1+/- and Npc1+/- mice fed either a low- or high-fat diet were determined at 70 days of age. An asterisk indicates a significant difference (P < 0.05) in weight when comparing Npc1+/- mice with Npc1+/- mice fed the same diet. Values are represented by means ± SEM (n = 15).
energy during the course of aging, regardless of whether mice were fed a low- or high-fat diet. Therefore, Npc1 haploinsufficiency was not associated with increased consumption of nutritional energy or hyperphagia when mice were fed either a low- or high-fat diet.

Magnetic resonance images for Npc1+/+ and Npc1−/− mice fed a low- (Fig. 3A) or a high-fat diet (Fig. 3B) at two different cross-sectional areas (spinal and abdominal) indicated the latter mice had increased adiposity. The Npc1+/− mice fed a low-fat diet had significantly increased body and abdominal fat (increased 30% from 10.9 to 14.2%, and 30% from 7.6 to 9.9%, respectively) compared with Npc1+/+ mice fed the same diet (Fig. 3C). Moreover, the Npc1+/− mice fed a high-fat diet had a significantly increased body and abdominal fat (increased 41.8% from 22.5 to 31.9%, and 36.0% from 12.8 to 17.4%, respectively) compared with Npc1+/+ mice fed the same diet (Fig. 3D). Therefore, Npc1 haploinsufficiency was associated with an increased percentage of body and abdominal fat when mice were fed either a low- or high-fat diet.

Representative images of gonadal adipose for Npc1+/+ and Npc1−/− mice fed a low- (Fig. 4A) or high-fat diet (Fig. 4B) indicated the latter mice have an increased average size of adipocytes. The Npc1+/− mice fed a low-fat diet had a modest but significantly increased average size of adipocytes (57% from 37.8 ± 1.5 to 59.4 ± 1.9 µm) compared with Npc1+/+ mice fed the same diet (Fig. 4C). Moreover, the Npc1+/− mice fed a high-fat diet had a modest but significantly increased average size of adipocytes (67% from 49.4 ± 1.6 to 82.7 ± 2.4 µm) compared with Npc1+/+ mice fed the same diet (Fig. 4D). Therefore, Npc1 haploinsufficiency was associated with adipocyte hypertrophy of gonadal adipose tissue when fed either a low- or high-fat diet.

Representative images of liver sections for Npc1+/+ and Npc1−/− mice fed a low- (Fig. 5A) or high-fat diet (Fig. 5B) indicated the latter mice have an increased amount of Oil Red O (ORO) staining. The Npc1+/− mice fed a low-fat diet had a significantly increased percentage of ORO-stained area (85% from 3.39 ± 0.18 to 6.32 ± 0.37%) compared with Npc1+/+ mice fed the same diet (Fig. 4C). Moreover, the Npc1+/− mice fed a high-fat diet had a significantly increased percentage of ORO-stained area (112% from 9.25 ± 0.59 to 19.59 ± 0.38%) compared with Npc1+/+ mice fed the same diet (Fig. 5D). Therefore, Npc1 haploinsufficiency was associated with hepatic steatosis when fed either a low- or high-fat diet.

The Npc1+/− mice fed either a low- or high-fat diet had a significantly increased concentration of liver triacylglycerol (260 and 63%, respectively) compared with Npc1+/+ mice fed the same diets, consistent with results presented in the previous section indicating a significantly increased percentage of ORO-stained area for liver sections (Table 1). However, there was no significant difference in the concentration of liver cholesterol or cholesteryl ester for Npc1+/+ and Npc1−/− mice fed either a low- or high-fat diet. Therefore, consistent with results presented in the last section indicating a significantly increased difference in the percentage of ORO-stained area for liver sections, Npc1 haploinsufficiency was associated with hepatic steatosis, but not altered sterol homeostasis when mice were fed either a low- or high-fat diet.

The Npc1+/− mice fed a low- or high-fat diet had a significantly increased concentration of plasma glucose (increased 18% from 7.3 ± 0.3 to 8.6 ± 0.3 mm, and 40% from 8.9 ± 0.4 to 12.5 ± 0.8 mm, respectively) compared with Npc1+/+ mice fed the same diets (Fig. 6A). Moreover, although there was no significant difference in the concentration of plasma insulin for Npc1+/+ and Npc1−/− mice fed a low-fat diet (0.37 ± 0.03 and 0.37 ± 0.04 nm, respectively), the Npc1+/− mice fed a high-fat diet had a significantly increased concentration of plasma insulin (increased 80.0% from 0.54 ± 0.03 to 0.97 ± 0.08 nm) compared with Npc1+/+ mice fed the same diet (Fig. 6B). Similarly, although there was no significant difference in the concentration of plasma leptin for Npc1+/+ and Npc1−/− mice fed a low-fat diet (0.07 ± 0.01 and 0.11 ± 0.02 nm, respectively), the Npc1+/− mice fed a high-fat diet had a significantly increased concentration of plasma leptin (increased 295% from 0.29 ± 0.04 to 1.15 ± 0.22 nm) compared with Npc1+/+ mice fed the same diet (Fig. 6C). Therefore, Npc1 haploinsufficiency was associated with significantly increased concentrations of plasma glucose, insulin and leptin when mice were fed a high-fat diet, suggesting that the Npc1 gene and high-fat diet interact to promote select metabolic features associated with insulin resistance.

The Npc1+/− mice fed a low-fat diet had a significantly increased concentration of blood glucose at each time point (18% at 0 min, 21% at 15 min, 27% at 30 min, 41% at 60 min and 61% at 120 min) during the glucose tolerance test compared with Npc1+/+ mice fed the same diet (Fig. 7A). Moreover, while the concentration of blood glucose for Npc1+/− mice fed a low-fat diet returned to the baseline level (0 time) after 120 min, the concentration of blood glucose for Npc1+/− mice fed a low-fat diet remained significantly increased (22% from 8.64 ± 0.28 to 10.59 ± 0.87 mm) after 120 min. The Npc1+/− mice fed a high-fat diet also had a significantly increased concentration of blood glucose at each time point (40% at 0 min, 20% at 15 min, 30% at 30 min, 28% at 60 min and 69% at 120 min) during the glucose tolerance test compared with Npc1+/+ mice fed the same diet (Fig. 7B). However, unlike the Npc1+/− mice fed a low-fat diet, the concentration of blood glucose for Npc1+/+ mice fed a high-fat diet remained significantly increased.
increased (39% from 8.92 ± 0.44 to 12.41 ± 1.37 mM) after 120 min, while the concentration of blood glucose for Npc1+/− mice fed a high-fat diet remained significantly more increased (68% from 12.49 ± 0.88 to 21.00 ± 1.83 mM) after 120 min. Therefore, Npc1 haploinsufficiency was associated with impaired fasting glucose and glucose intolerance when fed either a low- or high-fat diet.

The results indicated no significant difference in the concentration of cholesterol associated with different lipoproteins obtained from Npc1+/+ and Npc1+/− mice fed a low-fat diet. However, the results indicated a modest but significantly increased concentration of cholesterol associated with LDL and HDL (114 and 31%, respectively) for Npc1+/− mice when fed a high-fat diet (Table 2). Moreover, the results indicated a significantly increased concentration of triacylglycerol associated with HDL obtained from Npc1+/− mice fed either a low- or high-fat diet (56 and 107%, respectively), while there was no significant difference in the concentration of triacylglycerol associated with the other lipoproteins. Therefore, Npc1 haploinsufficiency was associated with an increased concentration of cholesterol and triacylglycerol with both LDL and HDL, consistent with metabolic or diabetic dyslipidemia.

**DISCUSSION**

We previously demonstrated that the BALB/cJ Npc1+/− mouse model, which possesses 50% decreased Npc1 gene dosage, is susceptible to increased weight gain when fed a high-fat diet, but not when fed a low-fat diet, consistent with a gene–diet interaction (17). To further investigate the Npc1

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**Figure 3.** Amount and distribution of body fat. Representative images for Npc1+/+ and Npc1+/− mice fed a low- (A) or high-fat diet (B) are provided at two different cross-sectional areas (spinal and abdominal) for each mouse at 70 days of age. The percentage of body and abdominal fat for Npc1+/+ and Npc1+/− mice fed a low- (C) or high-fat diet (D) was determined as described in the Materials and Methods section. An asterisk indicates a significant difference (P ≤ 0.05) in the percentage of fat when comparing Npc1+/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 5).
gene in relation to weight gain and metabolic features associated with insulin resistance, we interbred BALB/cJ Npc1+/− mice with wild-type C57BL/6J mice, the latter mouse strain commonly used to study aspects of diet-induced obesity and insulin resistance (18,19). In brief, the results from our study indicated that these Npc1+/− mice were susceptible to increased weight gain characterized by increased whole body and abdominal adiposity, adipocyte hypertrophy and hepatic steatosis in the absence of hyperphagia. Moreover, the Npc1+/− mice developed abnormal metabolic features

Figure 4. Size of adipocytes associated with adipose tissue. Representative images of adipocytes associated with gonadal adipose tissue for Npc1+/+ and Npc1+/− mice fed a low- (A) or high-fat diet (B) at 70 days of age are provided, where the bar represents a length equivalent to 100 μm. The percentage of cells in relation to cell diameter for Npc1+/+ and Npc1+/− mice fed a low- (C) or a high-fat diet (D) was determined as described in the Materials and Methods section. Values are represented by means ± SEM (n = 3).

Figure 5. Relative amount of neutral lipid associated with hepatocytes. Representative images of liver sections stained with ORO and counterstained with hematoxlin and eosin for Npc1+/+ and Npc1+/− mice fed a low- (A) or high-fat diet (B) at 70 days of age are provided, where the bar represents a length equivalent to 100 μm. The percentage of ORO-stained area for Npc1+/+ and Npc1+/− mice fed a low- (C) or high-fat diet (D) was determined as described in the Materials and Methods section. An asterisk indicates a significant difference (P ≤ 0.05) in the percentage of ORO-stained area when comparing Npc1+/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 3).
The concentration of liver triacylglycerol, cholesterol and cholesteryl ester for Npc1 haploinsufficiency considering that the hybrid C57BL/6J mice (20%) fed a high-fat diet (20). This result is consistent with other studies previously performed using the wild-type background, has been shown to be more resistant to diet-induced weight gain (21,22). With respect to the hybrid Npc1+/− mice, the results indicated a significantly increased body weight, gonadal adipose weight and liver weight when fed either a low- or high-fat diet compared with Npc1+/+ mice fed the same diets. Consistent with these results, the Npc1+/− mice fed either a low- or high-fat diet also had significantly increased whole body and abdominal adiposity compared with Npc1+/+ mice fed the same diets when measured using magnetic resonance imaging. In this regard, it is important to emphasize that the increased weight gain and adiposity associated with the Npc1+/− mice did not result from an increased consumption of food, thereby suggesting that Npc1 haploinsufficiency does not promote hyperphagia. This is in direct contrast to a number of other genetically susceptible obesity-related mouse models known to possess hormone signaling abnormalities effecting appetite control within the hypothalamus, including those causing deficiency of leptin (ob/ob mouse), the melanocortin-4 receptor (MC4r mouse) and the single-minded homolog 1 protein (Sim1 mouse) (23–25).

Consistent with increased body and abdominal adiposity, histological analysis of adipose and liver tissues indicated that Npc1+/− mice possessed both gonadal adipose hypertrophy and hepatic steatosis compared with Npc1+/+ mice fed either a low- or high-fat diet. It is interesting to note that for a number of years, the visceral-fat portal hypothesis has provided a putative explanation for the direct correlation between adipose hypertrophy and hepatic steatosis, whereby the enlarged and insulin resistant adipocytes were proposed to release excess free fatty acids into the portal blood, thereby resulting in subsequent triacylglycerol accumulation within liver hepatocytes (26,27). However, with the recent advent of more sophisticated stable-isotope labeling and monitoring techniques, studies now suggest that triacylglycerol accumulation within liver hepatocytes may serve a primary and central role in promoting both weight gain and metabolic dysfunction within peripheral tissues (28,29). This being the case, it is interesting to note that our early studies revealed that Npc1+/− mice possess hepatic steatosis in the absence of either being overweight or having adipocyte hypertrophy, even when fed a low-fat diet, thereby suggesting that Npc1 haploinsufficiency may specifically promote weight gain through altered hepatic lipid metabolism (30).

A number of studies have now indicated that hepatic steatosis and non-alcoholic fatty liver disease have a central role in

Table 1. Concentration of liver triacylglycerol, cholesterol and cholesteryl ester

<table>
<thead>
<tr>
<th></th>
<th>Low-fat diet</th>
<th>Npc1+/−</th>
<th>High-fat diet</th>
<th>Npc1+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol</td>
<td>25.2 ± 1.43</td>
<td>90.9 ± 12.2*</td>
<td>197.9 ± 10.3</td>
<td>323.3 ± 13.5*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>9.37 ± 0.32</td>
<td>9.97 ± 0.19</td>
<td>10.57 ± 0.28</td>
<td>9.83 ± 0.24</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>2.73 ± 0.10</td>
<td>2.36 ± 0.21</td>
<td>6.54 ± 0.65</td>
<td>8.71 ± 0.98</td>
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</tbody>
</table>

The concentration of liver triacylglycerol, cholesterol and cholesteryl ester for Npc1+/− and Npc1+/− mice fed either a low- or high-fat diet was determined at 70 days of age. An asterisk indicates a significant difference (P ≤ 0.05) in the concentration of lipid when comparing Npc1+/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 5).

Figure 6. Concentration of plasma glucose, insulin and leptin. The concentration of plasma glucose (A), insulin (B) and leptin (C) for Npc1+/+ and Npc1+/− mice fed either a low- or high-fat diet were determined at 70 days of age. An asterisk indicates a significant difference (P ≤ 0.05) in concentration when comparing Npc1+/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 15).

characterized by impaired fasting glucose, glucose intolerance, hyperinsulinemia, hyperleptinemia and dyslipidemia marked by an increased concentration of cholesterol and triacylglycerol associated with LDL and HDL. The overall results are consistent with a unique Npc1 gene–diet interaction in promoting both weight gain and metabolic features associated with insulin resistance.

As anticipated, the hybrid Npc1+/+ mice were modestly susceptible to diet-induced weight gain (11%) when compared with other studies previously performed using the wild-type C57BL/6J mice (20%) fed a high-fat diet (20). This result is reasonable considering that the hybrid Npc1+/− mouse,
promoting hepatic insulin resistance, which subsequently leads to whole body insulin resistance, in addition to the various associated metabolic abnormalities including type 2 diabetes mellitus and premature atherosclerosis (31–34). When compared with the Npc1+/+ mice, the results clearly indicated that Npc1−/− mice have impaired fasting glucose, glucose intolerance and increased plasma insulin/leptin levels when fed a high-fat diet, all of which serve as strong indicators for whole body insulin resistance and type 2 diabetes mellitus. Moreover, consistent with the development of hepatic steatosis, the lipid profile of Npc1−/− mice fed a high-fat diet was characterized by an increased concentration of cholesterol associated with both LDL and HDL, in addition to an increased concentration of triacylglycerol associated with LDL and HDL, in addition to an increased concentration of cholesterol within mouse tissues, including the liver (30,37). However, together our studies performed using either young or old mice fed either a low- or high-fat diet have now confirmed that Npc1−/− mice fed either a low- or high-fat diet was determined at 70 days of age. An asterisk indicates a significant difference (P ≤ 0.05) in concentration when comparing Npc1−/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 5).

The concentration and distribution of plasma cholesterol and triacylglycerol for Npc1+/+ and Npc1−/− mice fed either a low- or high-fat diet was determined at 70 days of age. An asterisk indicates a significant difference (P ≤ 0.05) in concentration when comparing Npc1−/− mice with Npc1+/+ mice fed the same diet. Values are represented by means ± SEM (n = 5).

Table 2. Concentration and distribution of plasma cholesterol and triacylglycerol

<table>
<thead>
<tr>
<th></th>
<th>Low-fat diet Npc1+/+</th>
<th>Npc1−/−</th>
<th>High-fat diet Npc1+/+</th>
<th>Npc1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>0.027 ± 0.005</td>
<td>0.035 ± 0.004</td>
<td>0.023 ± 0.006</td>
<td>0.043 ± 0.009</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.181 ± 0.007</td>
<td>0.214 ± 0.012</td>
<td>0.189 ± 0.013</td>
<td>0.239 ± 0.019</td>
</tr>
<tr>
<td>LDL</td>
<td>0.273 ± 0.017</td>
<td>0.324 ± 0.013</td>
<td>0.308 ± 0.028</td>
<td>0.659 ± 0.048*</td>
</tr>
<tr>
<td>HDL</td>
<td>2.371 ± 0.056</td>
<td>2.778 ± 0.220</td>
<td>2.701 ± 0.265</td>
<td>3.549 ± 0.083*</td>
</tr>
<tr>
<td>Total</td>
<td>2.851 ± 0.062</td>
<td>3.352 ± 0.248</td>
<td>3.221 ± 0.290</td>
<td>4.490 ± 0.125*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>0.203 ± 0.044</td>
<td>0.249 ± 0.034</td>
<td>0.147 ± 0.050</td>
<td>0.271 ± 0.065</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.957 ± 0.068</td>
<td>1.045 ± 0.049</td>
<td>0.870 ± 0.086</td>
<td>1.106 ± 0.124</td>
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<td>LDL</td>
<td>0.183 ± 0.007</td>
<td>0.175 ± 0.008</td>
<td>0.171 ± 0.021</td>
<td>0.180 ± 0.011</td>
</tr>
<tr>
<td>HDL</td>
<td>0.048 ± 0.004</td>
<td>0.075 ± 0.006</td>
<td>0.059 ± 0.009</td>
<td>0.122 ± 0.016*</td>
</tr>
<tr>
<td>Total</td>
<td>1.391 ± 0.111</td>
<td>1.542 ± 0.072</td>
<td>1.247 ± 0.139</td>
<td>1.679 ± 0.158</td>
</tr>
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</table>

Figure 7. Glucose tolerance test. A glucose tolerance test for Npc1+/+ and Npc1−/− mice fed either a low- (A) or high-fat diet (B) was performed at 70 days of age. The concentration of blood glucose at 0 min (fasting blood glucose) and after injection of a weight-adjusted bolus of glucose into the peritoneum at 15, 30, 60 and 120 min was determined. An asterisk indicates a significant difference (P ≤ 0.05) in concentration when comparing Npc1−/− mice with Npc1+/+ mice fed the same diet.
that the NPC1 protein has earlier been reported to function in the transport of fatty acids, but not cholesterol or cholesteryl esters, across associated membranes (5,40). Obviously, further studies will be required to more fully define the disparate functions that have now been attributed to the NPC1 protein.

In summary, the results have demonstrated that Npc1 haploinsufficiency promotes weight gain and metabolic features associated with insulin resistance, and therefore the encoded NPC1 protein has a previously unrecognized role in maintaining energy and metabolic homeostasis. These results are consistent with a recent genome-wide association study indicating that two NPC1 gene non-synonymous SNP variants (rs1805081 and rs1805082 encoding His215Arg and Ile858Val, respectively) are associated with common adult obesity and type 2 diabetes mellitus.

MATERIALS AND METHODS

Materials
The low-fat diet (14% kcal fat, Diet-7013) was purchased from Harlan Laboratories (Indianapolis, IN, USA) and the high-fat diet (45% kcal fat, Diet-07021302) was purchased from Research Diets (New Brunswick, NJ, USA). Free-Style Freedom Glucometer and test strips used to perform glucose tolerance tests were purchased from Abbott Diabetes Care (Alameda, CA, USA).

The Npc1 mouse model
A male BALB/cJ Npc1 heterozygous mouse (BALB/cJ Npc1+/−) and a female C57BL/6J normal mouse (C57BL/6J Npc1+/+) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). These mice were bred producing offspring that were subsequently interbred within a closed colony for approximately 20 generations, thereby resulting in a BALB/cJ–C57BL/6J hybrid Npc1 mouse strain. The BALB/cJ–C57BL/6J hybrid Npc1 mouse strain was produced to perform these studies since the C57BL/6J mouse strain has previously been reported to be more susceptible to weight gain, insulin resistance and development of hepatic steatosis when fed a high-fat diet compared with the BALB/cJ mouse strain (21,41,42). For the present study, 10 breeding pairs of mice (Npc1+/− and Npc1−/−) were established to generate male Npc1+/− and Npc1−/− offspring. After genotype analysis, the siblings were housed together (four mice per cage, two Npc1+/+ and two Npc1−/− whenever possible) to ensure identical housing environments. Moreover, an additional four cages were prepared to separately house Npc1+/− and Npc1−/− mice fed either a low- or high-fat diet to determine the consumption of food. The mice were initially maintained at the University of Arizona Animal Care Facility, and then subsequently at the University of New Mexico Health Sciences Center Animal Resources Facility in rooms with alternating 12 h periods of light and dark in accordance to regulations mandated by the Institutional Animal Care and Use Committee (IACUC).

Experimental design
The male mice were weaned at 21 days of age and genotype analysis was performed as previously described (16). The Npc1 mice were placed within cages and fed a low-fat diet until 30 days of age, at which time the mice were fed either a low- or high-fat diet until 70 days of age. At 70 days of age, the mice were fasted (4 h) in preparation for an intraperitoneal glucose tolerance test and/or sacrificed to harvest tissues. The mice were sacrificed using CO2 asphyxiation and the blood was obtained by cardiac puncture and collected into EDTA-treated tubes. The liver and intraperitoneal/gonadal adipose tissue were collected and weighed, with a small portion of each tissue frozen into OCT or fixed in 10% formalin for histological analyses. The remaining tissue was snap-frozen in liquid N2 and stored at −80°C for biochemical analyses.

Size of adipocytes
Excised mouse gonadal adipose tissue was stored in 10% formalin and then embedded in paraffin. The adipose tissue was cut into 10 μm sections and stained with hematoxylin and eosin. The sections were then viewed using an Olympus CKX41 inverted microscope (×10 magnification) and images were captured with an attached Olympus C-5050 digital camera (Olympus America, Center Valley, PA, USA). The images were processed using identical parameters with Adobe Photoshop software CS2 (Adobe Systems, Mountain View, CA, USA) and the diameter of adipocytes was manually determined from six random fields of adipose tissue obtained using three representative mice within each group based on body weight. The actual diameter of adipocytes was then determined using a hemocytometer as a calibrator.

Concentration of liver triacylglycerol, cholesterol and cholesteryl ester
Small portions of mouse liver were homogenized using a rotor stator homogenizer in hypotonic buffer (10 mM sodium phosphate pH 7.4 containing a complete protease inhibitor cocktail). The total lipids were extracted and separated by thin-layer chromatography using a hexane–diethylether–glacial acetic acid (80:20:1, v/v/v) solvent system as previously described (30). The mass of triacylglycerol, cholesterol and cholesteryl ester was determined using colorimetric assays (Infinity Triacylglycerol and Infinity Cholesterol, Thermo Electron Corporation, Pittsburg, PA, USA) and normalized to the corresponding protein that precipitated with organic solvent to provide the concentration of lipid.
Neutral lipid content of hepatocytes

A small portion of mouse liver was frozen into OCT, sectioned at a thickness of 10 µm and then stained for neutral lipid using ORO. The stained liver sections were examined and photographed using an Olympus IMT-2 inverted light microscope equipped with a ×20 objective (Olympus America) and Hamamatsu ORCA 100 grayscale CCD digital camera. The resulting neutral lipid staining was quantified using Simple PCI version 6.x software (Hamamatsu Corporation, Sewickley, PA, USA). In brief, the images of stained liver sections were overlaid with an intensity threshold and a watershed algorithm was used to separate nodes, discard noise (single threshold pixels) and quantify the amount of ORO staining. The relative amount of neutral lipid within hepatocytes was determined from 20 random fields of ORO-stained liver sections obtained using three representative mice within each group based on body weight.

Concentration and distribution of plasma cholesterol and triacylglycerol

The concentration and distribution of plasma cholesterol and triacylglycerol associated with lipoprotein classes were determined using high-sensitivity lipoprotein profiling by gel filtration HPLC performed by BioDyn Lifesciences (Santa Clara, CA, USA).

Magnetic resonance imaging

Magnetic resonance imaging was performed using a Bruker Biospec 7T MRI instrument (Billerica, MA, USA) with actively shielded gradients capable of 600 mT/m to non-invasively measure the percentage of body fat. Mice were anesthetized with 1.5% (v/v) isoﬂurane in O2 (1 l/min) and placed in a prone position within a 72 mm ID quadrature birdcage RF coil that was used for excitation and reception. In brief, the images of stained liver sections were overlaid with an intensity threshold and a watershed algorithm was used to separate nodes, discard noise (single threshold pixels) and quantify the amount of ORO staining. The relative amount of neutral lipid within hepatocytes was determined from 20 random fields of ORO-stained liver sections obtained using three representative mice within each group based on body weight.

Glucose tolerance test

A glucose tolerance test was performed on mice at 70 days of age to determine the presence of impaired glucose tolerance. In brief, after mice were fasted (4 h), a small drop of blood (2–3 µl) from the tail-tip was applied directly onto a glucometer test strip to obtain the baseline (0 min) fasting blood glucose reading. The mouse was then injected with a weight-adjusted bolus of sterile glucose saline solution (2 mg glucose/g body weight) into the peritoneum. After injection of the mouse, blood glucose was measured at 15, 30, 60 and 120 min. During the course of the glucose tolerance test, each mouse was housed in a separate cage with access to water, but not food.

Statistical analysis

For all experiments, quantitative data are represented as the mean ± standard error of the mean (SEM). Significant differences (P ≤ 0.05) between two groups of data were determined using the two-tailed Student’s t-test assuming equal variance.

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Conflict of Interest statement. None declared.

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