Published in final edited form as:

Eur J Lipid Sci Technol. 2012 March ; 114(3): 233–243. doi:10.1002/ejlt.201100309.

Beta-Cell Injury in Ncb5or-null Mice is Exacerbated by Consumption of a High-Fat Diet

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Summary

NADH-cytochrome b5 oxidoreductase (Ncb5or) in endoplasmic reticulum (ER) is involved in fatty acid metabolism, and Ncb5or^{-/-} mice fed standard chow (SC) are insulin-sensitive but weigh less than wild type (WT) littermates. Ncb5or^{-/-} mice develop hyperglycemia at about age 7 weeks due to β -cell dysfunction and loss associated with saturated fatty acid accumulation and manifestations of ER and oxidative stress. Here we report that when Ncb5or^{-/-} mice born to heterozygous mothers fed a high fat (HF) diet continue to ingest HF, they weigh as much as SC-fed WT at age 5 weeks. By age 7 weeks, diabetes mellitus develops in all HF-fed vs. 68% of SC-fed Ncb5or^{-/-} mice. Islet β -cell content in age 5-week Ncb5or^{-/-} mice fed HF for 7 days is lower (53%) than for those fed SC (63%), and both are lower than for WT (75%, SC, vs. 69%, HF). Islet transcript levels for markers of mitochondrial biogenesis (PGC-1 α) and ER stress (ATF6 α) are higher in Ncb5or^{-/-} than WT mice but not significantly affected by diet. Consuming a HF diet exacerbates Ncb5or^{-/-} β -cell accumulation of intracellular saturated fatty acids and increases the

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Conflict of Interest The authors have declared no conflict of interest.

frequency of ER distention from 11% (SC) to 47% (HF), thus accelerates β -cell injury in Ncb5or^{-/-} mice.

Keywords

diabetes; beta-cells; lipotoxicity; high-fat diet; ER stress

1 Introduction

Lipid metabolism plays an important role in pancreatic β -cell function and maintenance [1]. Diabetes mellitus (DM) results from β -cell dysfunction and loss [2,3] through autoimmune destruction of β -cells in Type 1 DM and Type 2 is associated with obesity and metabolic syndrome. The incidence of both forms of DM has risen dramatically in recent decades in association with increasing consumption of Western diets with high fat content. Dysregulation of lipid metabolism occurs in lean Type 1 patients prior to clinical manifestations [4], but the mechanism remains unclear.

Increased exposure to lipid, especially saturated fatty acids (SFA), leads to β -cell dysfunction that is manifested by impaired insulin production and secretion and eventually β -cell loss [3,5] in a process designated “lipotoxicity” [6]. Numerous studies of animal and cellular models of Type 2 DM suggest that lipotoxicity is mediated by endoplasmic reticulum (ER) stress and apoptosis [7–10].

We have generated a lean diabetic mouse model deficient in NADH-cytochrome b5 oxidoreductase (Ncb5or) [11]. Ncb5or is a multi-domain redox enzyme that is widely expressed in animal cells and appears to be associated with ER [12,13]. Disruption of the Ncb5or gene results in disappearance of Ncb5or protein from liver microsomes and pancreas and in altered cellular fatty acid metabolism [11,13–15]. Ncb5or^{-/-} mice develop hyperglycemia at about age 7 weeks [11]. Prediabetic Ncb5or^{-/-} mice exhibit β -cell dysfunction manifest by reduced islet insulin content, impaired glucose-stimulated insulin secretion, and glucose intolerance [11]. We have recently demonstrated that Ncb5or^{-/-} β -cell defects include increased intracellular free SFA levels and manifestations of ER and oxidative stress and that progression of ER distention correlates with the magnitude of β -cell dysfunction and loss in prediabetic Ncb5or^{-/-} mice [16].

Ncb5or^{-/-} mice exhibit lower body weights than wild-type (WT) littermates and reduced body fat stores (lipotrophy) [15]. Livers of prediabetic Ncb5or^{-/-} mice at age 5 weeks exhibit lower triacylglycerol (TAG) content and reduced levels of monounsaturated fatty acids (MUFA) relative to SFA (MUFA/SFA) in neutral lipids. The latter is an index of the action of stearoyl-CoA desaturases (SCD) that convert palmitic and stearic acids to palmitoleate and oleate, respectively [15]. Ncb5or^{-/-} hepatocytes display reduced SCD specific activity and accumulate higher levels of SFA in the free fatty acid (FFA) pool, which promotes increased fatty acid catabolism and oxidative stress [15]. The pathogenesis of lipotrophy in Ncb5or^{-/-} mice appears to be independent of diabetes because Ncb5or^{-/-} mice that receive transplants of WT islets are normoglycemic at least through age 12 weeks but nonetheless have reduced hepatic TAG content and body fat [14]. When Ncb5or^{-/-} pups are fed a high fat (HF) diet for 10 days starting immediately after weaning, their hepatic TAG content normalizes but their body weight remains lower than WT littermates [15]. Because these HF-fed Ncb5or^{-/-} mice do not develop hyperglycemia by age 4.5 weeks, it has been considered possible that long-term HF diet consumption might reverse lipotrophy without adversely affecting glucose tolerance or β -cell function in Ncb5or^{-/-} mice.

Here we report results of studies to evaluate that possibility. We have examined effects of HF-feeding on body weight and β -cell function in lean *Ncb5or^{-/-}* mice and find that this intervention results in partial restoration of body weight of *Ncb5or^{-/-}* mice but also in accelerated development of diabetes. This process is associated with HF diet-induced exacerbation of saturated fatty acid accumulation in intracellular lipid pools, ER distention, and β -cell loss.

2 Material and methods

2.1 Materials

Chemicals for electron microscopy from Electron Microscopy Sciences (Hatfield, PA) and oligonucleotides from Integrated DNA Technologies (Coralville, IA). Other reagents and culture media were from Sigma (St. Louis, MO) except otherwise specified.

2.2 Animals and diet

The *Ncb5or^{-/-}* line of mice was generated as previously described [11] and backcrossed into BALB/c for over 12 generations. The animal experimentation protocol was approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Mice were handled in accordance with National Institutes of Health guidelines. Heterozygous breeders were used to generate male animals for experiments. All were housed in a pathogen-free facility on a 12-hr light/dark cycle with *ad libitum* access to water and standard chow (SC, TD8604 from Harlan Teklad, Madison, WI) or a high fat (HF) diet (F5194 from BioServ, Frenchtown, NJ) as previously described [15]. The SC diet contains 3.8 kcal/g, 4% fat (in the form of TAG) by weight, and its fatty acid composition 16% SFA, 24% MUFA, and 60% polyunsaturated fatty acids (PUFA). The HF diet contains 5.1 kcal/g, 35% fat by weight, and its fatty acid composition is 40% SFA, 50% MUFA, and 10% PUFA. Long-term HF feeding was initiated with pregnant dams and continued in weaned pups. The genotype of the dams was heterozygous *Ncb5or^{+/-}*, and their ages were between 8 and 16 weeks when administration of the HF diet was begun. These dams did not develop glucose intolerance or hyperglycemia during pregnancy or while nursing their pups over the 5–6 week period of study. Short-term HF loading started at age 4 weeks and lasted for 3 or 7 days. BALB/c mice were chosen in this study due to their higher sensitivity to HF loading than C57BL/6 mice, which were used in recent studies [15,16].

2.3 Blood glucose measurement and glucose tolerance tests

Tail vein whole blood glucose levels were measured with an *OneTouch* Ultra glucometer (LifeScan, Milpitas, CA). Glucose tolerance tests (GTT) were performed as previously described [11,16]. Areas under the curve (AUC) for blood glucose readings in GTTs were calculated as described [17] and expressed in arbitrary units.

2.4 Immunohistochemistry

Formalin-fixed paraffin-embedded pancreata were used for hematoxylin/eosin (H & E) staining, and immuno-staining for insulin and glucagon was performed with the Histostain-Plus RABBIT-DAB kit and antibodies (Invitrogen, Carlsbad, CA) as previously described [16].

2.5 Electron microscopy

Transmission electron microscopy (TEM) of islets was performed as previously described [16]. Briefly, nucleus-positive β -cells were examined for the frequency and severity of distended ER in a double-blind fashion (3–4 islets per sample, 3 mice per group). Digital islet maps were constructed from the same TEM grid to assess β -cell and non- β -cell

contents. Fifty to 500 nucleus-positive cells per islet were counted and categorized based on the distinct morphology of their granules. Numbers of β - and non- β -cells (three islets per mouse) were used to calculate the percentages of these cell types.

2.6 Islet isolation

Islets were isolated with a collagenase infiltration method as previously described [16] and stored as pellets at -80°C until use.

2.7 Quantitative RT-PCR (qRT-PCR)

Each total RNA sample for reverse transcription and qRT-PCR was prepared from 500 islets from prediabetic Ncb5or $^{-/-}$ or WT mice as previously described [16]. Transcript levels for target genes were obtained by normalizing against 18S rRNA (internal control). Primer sequences are available upon request.

2.8 Lipid profiling

Lipid profiling of isolated islets from each mouse was performed as previously described [16]. Briefly, islets were sonicated for 10 one-second pulses in ice-cold lysis buffer (18 mM Tris-HCl, 300 mM Mannitol, 50 mM EGTA, pH 7.6) in the presence of protease inhibitors. Triacylglycerol (TAG), free fatty acids (FFA), and phospholipids (PL) were isolated by thin layer chromatography and converted to fatty acid methyl esters (FAME) before analyses on a Varian gas chromatography-mass spectrometry (GC-MS) system. Individual fatty acid species were quantified relative to internal standards of heptadecanoic acid (C17:0), triheptadecanoin (17:0/17:0/17:0-TAG) (NuCheck Prep, Elysian, MN), and 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (17:0/17:0-GPE, Sigma), as previously described [15]. The resultant values were used to calculate the sum of total FAs and their desaturation index (DI). Lipid content was normalized to measured sample protein content, which was determined with BCA reagent (Pierce, Rockford, IL). Supelco 37 FAME standards (Sigma) were used to identify FAME species by relative retention times on GC analyses.

2.9 Statistical analysis

Levels of significance for differences among groups were determined using Analysis of Variance (ANOVA). A p value <0.05 was considered statistically significant. Standard errors of the mean are displayed in the figures.

3 Results

3.1 Long-term HF diet consumption partially restores body weight but accelerates development of diabetes in Ncb5or $^{-/-}$ mice

Ncb5or $^{-/-}$ mice gained weight more slowly and achieved lower maximal body weights than WT mice when fed standard chow (SC) (Fig. 1A). To determine whether this lean phenotype can be corrected by increased dietary fat intake, a lard-based high fat (HF) diet was fed to pregnant Ncb5or $^{+/+}$ dams and then to their weaned pups. Rates of weight gain and maximal weights were greater in HF-fed WT and Ncb5or $^{-/-}$ mice compared to SC-fed counterparts. From birth to age 5 weeks, body weights of HF-fed exceeded those of SC-fed Ncb5or $^{-/-}$ mice and were similar to those of SC-fed but lower than those of HF-fed WT mice (Fig. 1A). HF-fed Ncb5or $^{-/-}$ mice achieved maximal body weights at about age 5 weeks and thereafter maintained stable weights, but WT mice continued to gain weight until about age 11 weeks (Fig. 1A).

HF-fed Ncb5or^{-/-} mice developed hyperglycemia at an earlier age than did SC-fed counterparts (Fig. 1B). About 50% of HF-fed Ncb5or^{-/-} mice exhibited hyperglycemia at age 5.5 weeks, and this occurred at about age 6.5 weeks for SC-fed counterparts, which indicates that the HF diet accelerated the onset of diabetes by about one week (Fig 1B). By age 7 weeks, hyperglycemia occurred in all Ncb5or^{-/-} mice fed HF vs. 68% of those fed SC.

3.2 Short-term HF diet consumption accelerates Ncb5or^{-/-} β -cell dysfunction

We have recently demonstrated that SC-fed Ncb5or^{-/-} mice first exhibit β -cell dysfunction at age 5 weeks manifest by morphological abnormalities that include ER distention, reduced β -cell size, and increased mitochondrial content [16]. We thus examined effects of consuming a HF diet for 3 or 7 days by Ncb5or^{-/-} mice that had been fed SC from birth to age 4 weeks.

Before the HF dietary intervention, SC-fed Ncb5or^{-/-} mice exhibited blood glucose levels and glucose tolerance test (GTT) curves that are similar to those of SC-fed WT mice (Fig. 2A). After consuming the HF diet for 3 days, the glucose tolerance of WT mice was significantly impaired relative to their SC-fed counterparts ($p < 0.05$ for all time-points, Fig. 2A), and the GTT area under the curve (AUC) increased significantly for the HF-fed WT mice. The GTT AUC also rose for Ncb5or^{-/-} mice fed the HF diet for 3 days, but this did not reach statistical significance ($p = 0.12$). No significant differences in AUC between genotypes were observed for mice fed SC or the HF diet for 3 days (Fig. 2B). This is consistent with similar fasted and fed blood glucose levels for WT and Ncb5or^{-/-} mice that had consumed the HF diet for 3-days. Under these conditions, fasting blood glucose levels were 180 ± 13 (n = 10) vs. 176 ± 9 (n = 6) mg/dL for WT and Ncb5or^{-/-} mice, respectively. The corresponding fed blood glucose values were 178 ± 4 vs. 225 ± 10 mg/dL for WT and Ncb5or^{-/-} mice, respectively. After consuming the HF diet for 7 days, WT mice exhibited a small but significant increase in GTT AUC, while Ncb5or^{-/-} mice exhibited a large and significant increase that reflected marked deterioration in glucose tolerance compared to SC-fed counterparts (Fig. 2B). Ncb5or^{-/-} mice developed higher blood glucose values than WT throughout the GTT, but the difference was significant ($p < 0.05$) only at 15 min (Fig. 2A). Similarly, the AUC was higher for Ncb5or^{-/-} than WT mice fed the HF diet for 7 days, but the difference failed to achieve significance ($p = 0.17$) because of large variations among Ncb5or^{-/-} mice. Overt hyperglycemia did develop in 33% of Ncb5or^{-/-} fed the HF diet for 7 days (data not shown).

The above experiments were performed with Ncb5or^{-/-} mice and WT littermates on a BALB/c genetic background. In contrast, consumption of a HF diet for 7 days or more did not affect the onset of diabetes in C57BL/6 Ncb5or^{-/-} mice (not shown). All subsequent experiments were performed with SC- and HF-fed BALB/c mice for comparison to each other and to those with SC-fed C57BL/6 mice [16].

3.3 Consumption of a HF diet accelerates β -cell loss in Ncb5or^{-/-} mice

Histologic examination of pancreatic islet sections from Ncb5or^{-/-} mice fed a HF diet for 7 days (Fig. 3A) revealed “bubbly” cells, some containing nuclei and cytoplasm. Similar structures are observed in islets of SC-fed prediabetic Ncb5or^{-/-} mice at age 6 weeks, not 5 weeks [16]. These structures do not stain with Oil Red O (not shown) and thus appear not to represent accumulations of neutral lipids. They also do not appear to represent vascular cells, and no red blood cells are observed. Rather, these structures appear to correspond to moribund β -cells that contain severely distended ER, as described further below.

These islets were also examined by immuno-fluorescence microscopy using antibodies against insulin (Fig. 3B) and glucagon (Fig. 3C) to identify β -cells and α -cells, respectively.

Of 5-week-old mice, islets from HF-fed Ncb5or^{-/-} mice contained significantly fewer insulin-positive β -cells than did those from SC-fed counterparts [16]. No such difference was observed in WT islets from SC- or HF-fed mice. Compare to WT counterparts, islets from HF-fed Ncb5or^{-/-} mice had disproportionally more glucagon-positive α -cells, indicating β -cell loss. Fewer than 25% of total islet cells (equivalent to fewer than 50% of remaining β -cells, see below) of HF-fed Ncb5or^{-/-} mice contained immuno-reactive insulin (Fig. 3B). Compared to SC-fed counterparts [16], islets of HF-fed Ncb5or^{-/-} mice contained significantly more insulin- and glucagon-deficient cells, which appear to be degranulated β -cells (see below).

3.4 Consumption of a HF diet exacerbates ER distention in Ncb5or^{-/-} β -cells

Transmission electron microscopic (TEM) analyses of islets from SC-fed Ncb5or^{-/-} mice revealed distended ER structures (dERs) in β -cells at age 5 and 6 weeks (Fig. 4B,C, respectively). These dERs are vacuole-like structures that represent swollen ER sacs and indistinguishable from the structures previously observed in C57BL/6 Ncb5or^{-/-} mice [16]. No dER was observed in WT β -cells from SC-fed mice (Fig. 4A), although HF-fed WT mice contained mildly dilated rough ER (rER) (Fig. 4D).

Islets from HF-fed Ncb5or^{-/-} mice at age 5 weeks (Fig. 4E) displayed more abundant and more severely distorted dER in β -cells than did age-matched and even older SC-fed Ncb5or^{-/-} mice (Fig. 4B,C). Some dERs have ribosome-like particles attached to membrane surfaces that are visible at high magnification (indicated by “*” in Fig. 4G), similar to those particles on the surface of rough ER (rER) in WT β -cells (indicated by “*” in Fig. 4F). The dER-containing Ncb5or^{-/-} β -cells displayed significantly fewer insulin granules than those without dER as previously reported [16]. There was no discernible alteration in β -cell mitochondrial morphology in islets from 7 day HF-fed or SC-fed Ncb5or^{-/-} mice, similar to our previous findings [16].

We scored ER-stress in β -cells by the percentage of dER-containing β -cells among the total β -cell population. As illustrated in Fig. 5A, the frequency of dER among β -cells increased from 11.4 $\pm$ 1.7% in SC-fed to 47.0 $\pm$ 4.0% in HF-fed Ncb5or^{-/-} mice at age 5 weeks, and the latter is even higher than that in SC-fed mice at age 6 weeks (34.5 $\pm$ 2.0%).

Loss of islet β -cell content was assessed by counting β -cells and non- β (α -, δ -, and PP) cells that are readily distinguishable based on characteristics of their secretory granules. At age 5 weeks, HF-fed Ncb5or^{-/-} mice displayed lower islet β -cell content (53.0 $\pm$ 1.3%) than that of SC-fed Ncb5or^{-/-} mice of the same age (63.2 $\pm$ 3.2%) and even those of one week older (57.5 $\pm$ 3.5%). These values are significantly lower than those in WT counterparts, i.e., 75.3 $\pm$ 0.5% (5 weeks) and 72.0 $\pm$ 1.7% (6 weeks) in SC-fed and 69.3 $\pm$ 3.2% in HF-fed mice (Fig. 5B). The decrease in islet β -cell content that is associated with the consumption of HF in Ncb5or^{-/-} mice is greater than that due to the aging in SC-fed BALB/c and C57BL/6 counterparts [16]. Severe degranulation was observed in the majority of surviving β -cells in HF-fed Ncb5or^{-/-} mice, which is consistent with our observations stated above. These results suggest that loss of islet β -cells and ER stress in surviving β -cells are accelerated significantly when Ncb5or^{-/-} mice consume a HF diet.

3.5 Consumption of a HF diet for 3 days exacerbates lipid accumulation in Ncb5or^{-/-} islets

We next examined the effects of consuming a HF diet for 3 days on the lipid content of islets from prediabetic Ncb5or^{-/-} mice at age 4.5 weeks. At this age there is no difference between genotypes in GTT (Fig. 2). Fatty acid profiles of intracellular FFA, TAG, and PL in isolated islets were determined by GC/MS after transmethylation to FAME. Similar measurements were not possible after seven days of HF consumption because poor yields of

islets were obtained from *Ncb5or*^{-/-} mice, which reflect severe β -cell loss and islet fragility under these conditions.

We observed no significant differences between *Ncb5or*^{-/-} and WT mice in intracellular PL content or PL fatty acid composition in isolated islets after consumption of a HF diet (Table 1). For both genotypes the amounts of intracellular FFA were 5–6 fold lower than those of PL. *Ncb5or*^{-/-} islets contained significantly higher levels (*ca.* 1.5-fold) of saturated FFA compared to WT, but the two genotypes displayed no significant differences in the levels of monounsaturated FFA or desaturation indices (DI, MUFA:SFA ratio) of 16- and 18-carbon species. We have previously reported similar findings with islets from SC-fed *Ncb5or*^{-/-} and WT mice [16].

Islet contents of intracellular TAG were only 20–25% of those for FFA for both genotypes after 3 days of consumption of a HF-diet, but total islet TAG content was significantly higher for *Ncb5or*^{-/-} mice compared to WT (Table 1). Among specific TAG fatty acid substituents, amounts of SFA ($p = 0.03$) and MUFA ($p = 0.02$) in *Ncb5or*^{-/-} islet TAG significantly exceeded those for WT. Palmitate was the most abundant SFA and oleate the most abundant MUFA in *Ncb5or*^{-/-} islet TAG (Table 1). In contrast, no significant difference in islet TAG content was observed previously for SC-fed *Ncb5or*^{-/-} and WT mice, although there was a non-significant trend ($p = 0.11$) for higher values in *Ncb5or*^{-/-} mice [16]. There is also a non-significant trend for desaturation indices (DI) for islet TAG fatty acid substituents to be higher for *Ncb5or*^{-/-} than for WT mice fed either a HF diet (Table 1) or SC [16].

3.6 Consumption of a HF diet for 3 days results in increased expression of ER-stress response genes and mitochondrial biogenesis marker in islets from *Ncb5or*^{-/-} mice

To explore mechanisms whereby consumption of a high fat diet exacerbated islet lipid accumulation, induction of ER stress, and β -cell loss in *Ncb5or*^{-/-} mice, we examined dietary effects on expression of transcripts for various genes involved in these processes (Table 2).

Islet expression of insulin (*Ins2*) transcripts did not differ between WT and *Ncb5or*^{-/-} fed SC or the HF diet (Table 2). In contrast, islet glucagon (*Gln*) transcript levels were over 2-fold higher for *Ncb5or*^{-/-} than for WT mice when fed SC or after 3 days of consuming a HF diet. The dietary intervention itself did not affect insulin and glucagon transcript levels significantly (Table 2). The reduced insulin:glucagon ratio is consistent with lower islet β -cell content observed for *Ncb5or*^{-/-} mice compared to WT for mice fed either SC or the HF diet (Fig. 5B).

With respect to islet ER stress marker transcripts, levels of ATF6 α were significantly higher for SC-fed *Ncb5or*^{-/-} compared to WT, but levels of ATF3 and CHOP were similar between the two genotypes (Table 2), which mimics the pattern previously observed with C57BL/6 mice [16]. In contrast, higher islet transcript levels for Xbp1s (active spliced form), BiP and Grp94 were observed for *Ncb5or*^{-/-} than WT mice on the C57BL/6 background [16], but there was no difference between genotypes in islet levels of these transcripts for BALB/c mice (Table 2), reflecting a less robust ER stress response for that genetic background. After 3 days of consuming the HF diet, *Ncb5or*^{-/-} islet CHOP transcript levels exceeded those of SC-fed counterparts, while ATF6 α and Xbp1s transcript levels were lower in islets of HF-fed *Ncb5or*^{-/-} mice compared to those of SC-fed counterparts. In contrast, consumption of the HF diet for 3 days by WT mice resulted in a significant decline only in islet ATF6 α transcript levels compared to SC-fed counterparts. After 3 days' consumption of the HF diet, ATF6 α and ATF3 transcript levels were higher in *Ncb5or*^{-/-} islets than in WT, but

transcript levels for all other examined ER stress markers were similar between the two genotypes (Table 2).

Genes involved in fatty acid metabolism that were examined include PGC-1 α (Peroxisome Proliferator-Activated Receptor γ -Coactivator, a marker of mitochondrial biogenesis), FAS (Fatty Acid Synthase, the enzyme responsible for *de novo* synthesis of palmitate), SCD-1 and -2 (stearoyl-CoA desaturase isoforms 1 and 2, which convert the saturated fatty acids palmitate and stearate to the monounsaturated fatty acids palmitoleate and oleate, respectively), CD36 (a translocase that imports fatty acids into cells), and LPL (lipoprotein lipase, which hydrolyzes triglycerides in lipoproteins to release free fatty acids and monoacylglycerol and promotes fatty acid uptake) (Table 2).

Of the above genes involved in fatty acid metabolism, islets of SC-fed Ncb5or^{-/-} mice expressed significantly higher transcript levels for PGC-1 α , LPL, and SCD2 compared to WT (Table 2), and this is similar to findings in C57BL/6 mice (W.F. Wang and H. Zhu, unpublished results). Consuming the HF diet for 3 days caused transcript levels to fall for SCD1, SCD2, and LPL in Ncb5or^{-/-} mice and for transcript levels of SCD1 and LPL to fall in WT mice. This resulted in statistically indistinguishable levels of transcripts between the genotypes for most of these fatty acid metabolism genes. The only exception among them is PGC-1 α , whose transcript levels were unaffected by the HF diet and remained 3-fold higher for Ncb5or^{-/-} than for WT mice (Table 2).

Discussion

Our findings here indicate that, compared to WT, islets of prediabetic BALB/c Ncb5or^{-/-} mice display severe ER distention, express higher transcript levels for the mitochondrial biogenesis marker PGC-1 α , and contain higher intracellular levels of saturated FFA. We have previously reported similar findings for mice on a C57BL/6 genetic background [16], and these effects of Ncb5or-deficiency thus appear to be independent of genetic background.

We have also observed here that consumption of a HF diet increases body weight of BALB/c Ncb5or^{-/-} mice toward normal WT levels and exacerbates β -cell injury and accumulation of saturated fatty acids in FFA and TAG pools of Ncb5or^{-/-} islets. Consumption of a HF diet restores hepatic TAG content in C57BL/6 Ncb5or^{-/-} mice to the same level of WT after 10 days but does not accelerate diabetes onset or restore body weight [15]. These findings indicate that consumption of a HF diet produces more exaggerated deleterious effects in Ncb5or^{-/-} mice on the BALB/c compared to the C57BL/6 genetic background. This is consistent with other reports that C57BL/6 mice are less sensitive than BALB/c mice to adverse consequences of HF diets, such as expression of agouti-related peptide (AgRP) in brain [18] or severity of diabetes in *ob/ob* mice [19].

Increased accumulation of saturated FFA in Ncb5or^{-/-} islet β -cells occurs in mice fed either SC or HF diet, and this appears to trigger increased PGC-1 α expression and mitochondrial biogenesis in β -cells, and ultimately β -cell dysfunction [16]. Others have shown that increased PGC-1 α expression is functionally associated with suppression of β -cell bioenergetic function and with reductions in glucose-induced rises in β -cell [ATP], effects on membrane electrical activity, Ca²⁺-influx, and insulin secretion [20]. Increased mitochondrial capacity for fatty acid oxidation has been shown to attenuate SFA-induced lipotoxicity in β -cells [21]. It is likely that β -cells of Ncb5or^{-/-} mice are chronically subjected to excessive levels of SFA, and this may contribute to development of their abnormalities in mitochondrial content and morphology [11], increased production of reactive oxygen species and hypersensitivity to oxidative stress [16].

Consumption of a HF diet for 3 days results in increased accumulation of intracellular TAG in islets of *Ncb5or*^{-/-} mice, although their hepatic TAG content remains below that of WT (W. Wang and H. Zhu, unpublished results). Lipogenesis in insulin-sensitive tissues appears to be increased in *Ncb5or*^{-/-} mice, as reflected by their increased body weight, and this is likely to be associated with insulin resistance that exacerbates adverse effects of their intrinsic β -cell defects on glucose tolerance.

Similar to BALB/c counterparts, SC-fed C57BL/6 *Ncb5or*^{-/-} mice accumulate higher islet transcript levels compared to WT for LPL and SCD2, which act to increase fatty acid availability and to produce monounsaturated from saturated fatty acids, respectively, as well as genes involved in TAG synthesis (W.F. Wang and H. Zhu, unpublished results). HF ingestion in BALB/c *Ncb5or*^{-/-} mice does not change islet PGC-1 α transcript expression, which is higher than WT for mice fed either SC or the HF diet, but does reduce SCD1, SCD2 and LPL transcript levels to a larger extent in *Ncb5or*^{-/-} mice than that in WT. This likely reflects the saturated capacity of mitochondrial fatty acid oxidation and of fatty acid conversion in β -cells of *Ncb5or*^{-/-} mice and the diversion of excess FFA into TAG biosynthetic pathways. Increased capacity of TAG synthesis has been associated with attenuation of SFA-induced lipotoxicity [22,23], but it does not appear to be sufficient in *Ncb5or*^{-/-} islets that are constantly exposed to excess saturated SFA.

Increased accumulation of SFA in FFA and TAG in *Ncb5or*^{-/-} islets is associated with severe ER distention. This structure resembles the disruption of ER membrane structure and integrity produced in rat pancreatic β -cell line BRIN-BD11 [10] and CHO cells [24] that are incubated with excess palmitate. Palmitate-treated CHO cells accumulate SFA in ER TAG and phosphatidylcholine pools, but not FFA [24]. *Ncb5or*^{-/-} and WT islets display nearly identical fatty acid profiles of total cellular PLs. The latter has also been observed in islets of Zucker diabetic fatty (ZDF) rat, a common lipotoxic diabetes model [25]. It remains unclear whether SFA accumulate differentially in various subcellular compartments or lipid classes in *Ncb5or*^{-/-} islets because our current extraction protocol does not distinguish among them. We are now attempting to examine these issues with much larger numbers of islets subjected to subcellular fractionation procedures to isolate plasma membranes, mitochondria, and microsomes, which will be analyzed separately to determine their lipid compositions.

Our previous studies suggest that *Ncb5or* may function as an electron donor for the SCD reaction to convert saturated to monounsaturated fatty acids [15]. The SCD index is computed from the relative amounts of SFA and MUFA in a given fatty acid pool and is taken to reflect action of the SCD enzyme *in vivo*. Prediabetic *Ncb5or*^{-/-} mice exhibit intracellular TAG SCD indices in hepatocytes that are lower than WT and also have lower hepatic microsomal SCD specific enzymatic activity [15]. Islets of HF-fed *Ncb5or*^{-/-} mice contain increased SFA levels in intracellular FFA and TAG pools compared to WT, but the SCD indices of islet lipid pools and islet SCD transcript levels do not differ between HF-fed WT and *Ncb5or*^{-/-} mice. SCD has been implicated in cytoprotective mechanism against SFA-induced lipotoxicity [23,26]. With *ob/ob* mice, disruption of the SCD1 gene accelerates onset of diabetes and is associated with increased islet content of intracellular FFA and TAG and with an increased SFA content of islet lipid pools [27]. Deficiency of SCD1 and of *Ncb5or* result in similar changes in islet total lipid content, although reduction of SCD indices is more marked with SCD-null *ob/ob* mice compared to HF-fed *Ncb5or*^{-/-} mice. These issues require further scrutiny, but existing information does suggest that *Ncb5or* and SCD1 are functionally related in β -cells and that *Ncb5or* could serve as a non-exclusive redox partner in the SCD1 reaction.

Short term consumption of a HF diet results in increased transcript levels for the ER stress response gene ATF3 in islets of *Ncb5or*^{-/-} mice compared to WT, which is consistent with

the fact that ATF3 protects β -cells from lipid-overload [28]. HF ingestion, however, results in reduction of transcript levels for ATF6 α and Xbp1s, both of which are known to promote ER lipid synthesis in response to ER stress [29–31]. Increased expression of ATF3 and ATF6 α in Ncb5or^{-/-} β -cells might represent compensatory responses to mitigate adverse effects of chronic lipid stress. Together, our findings strongly suggest that consumption of a high fat diet accelerates the development of β -cell dysfunction and loss in Ncb5or-null mice by exacerbating lipid overload that triggers ER stress and that Ncb5or is an important component of β -cell defense mechanisms to attenuate lipotoxic effects of saturated fatty acids.

Acknowledgments

We are grateful to Dr. H. Franklin Bunn (Brigham and Women's Hospital) for generous support. We thank colleagues at the University of Kansas Medical Center for suggesting the high-fat dietary intervention studies (Grace Guo), for providing training in islet isolation (S. Janette Williams), and for assistance with electron microscopy (Barbara Fegley). This work is supported by NIH grant RO1-DK067355 (H.F.B. and H.Z.). Y.G. is supported by a fellowship from the China Scholarship Council. S.E.C. is supported by NIH R01-HD047315. J.T. is supported by NIH R37-DK34388, P41-524 RR00954, P60-DK20579, and P30-DK56341. Core facilities at KUMC are supported by NIH HD002528.

Abbreviations

dER	distended endoplasmic reticulum
DI	desaturation index
DM	diabetes mellitus
ER	endoplasmic reticulum
FA	fatty acids
GTT	glucose tolerance test
NADH	nicotinamide adenine dinucleotide (reduced)
PL	phospholipids
SFA	saturated fatty acids
TEM	transmission electron microscopy
UPR	unfolded protein response
WT	wild-type.

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Practical applications

Lipid-induced beta-cell injury plays a key role in the etiology of Type 2 diabetes in association with metabolic syndrome and obesity. We have established a lean diabetes mouse model lacking Ncb5or, a redox enzyme that functions in cellular fatty acid metabolism by assisting desaturases to convert saturated to monounsaturated fatty acids. Ncb5or-null mice develop early-onset diabetes as a result of beta-cell dysfunction and loss, which is associated with intracellular accumulation of saturated free fatty acids and elevated markers of ER and oxidative stress in beta-cells. Consumption of a high fat diet partially restores body weight of Ncb5or-null mice but accelerates onset of diabetes by exacerbating beta-cell lipid overload and ER stress. This mouse model simulates lipid-induced beta-cell injury in Type 2 diabetes and allows us to elucidate the role of fatty acid metabolism in beta-cell function and maintenance.

FIG. 1. (A) Gross body weight of *Ncb5or*^{-/-} and WT mice and (B) frequency of overt diabetes in *Ncb5or*^{-/-} mice fed with standard chow (SC) or a high fat (HF) diet

Mean body weight ± SE (standard error of the mean) is displayed as a function of age till 13 weeks of age. For each data point, N>20 (SC) or N>11 (HF). WT is denoted by “+/+” and *Ncb5or*^{-/-} is denoted by “-/-”.

FIG. 2. Glucose Tolerance Tests (GTT) for *Ncb5or*^{-/-} and WT mice at age 5 weeks that were fed standard chow (SC) or that consumed a high fat (HF) diet for 3 or 7 days

(A) For SC diet, n = 7 for WT and n = 9 for *Ncb5or*^{-/-} mice. For 3d HF diet, n = 10 for WT and n = 6 for *Ncb5or*^{-/-} mice. For 7d HF diet, n = 7 for WT and n = 7 for *Ncb5or*^{-/-} mice. WT is denoted by “+/+” and *Ncb5or*^{-/-} is denoted by “-/-”. (B) Area under the curve for blood glucose readings of each condition. An asterisk (*) denotes a significant difference ($p < 0.05$).

FIG. 3. Histologic and immunohistochemical staining for insulin and glucagon in pancreata of 5 week-old WT and Ncb5or^{-/-} mice after consuming a high fat (HF) diet for 7 days

Panels *A* and *D* represent staining with hematoxylin and eosin (H & E); panels *B* and *E* immuno-staining for insulin; panels *C* and *F* immuno-staining for glucagon. The 'bubbly' cells (marked with asterisks (*)) in *D–F* were observed in all ($n = 15$) pancreatic sections examined from Ncb5or^{-/-} mice. Immunostaining for insulin in Ncb5or^{-/-} sections involved longer exposure time than for WT (35 min. vs. 15 min.) during color development. Insulin- and glucagon-negative cells observed in Ncb5or^{-/-} islets (*C* and *F*) likely represent degranulated β -cells. Glucagon-positive α -cells occupied a greater fraction of total islet area in Ncb5or^{-/-} islets compared to WT.

FIG. 4. Ultrastructural images of β -cells from WT and $Ncb5or^{-/-}$ mice

Transmission electron microscopic (TEM) images of β -cells from 5-week-old SC-fed WT (A) and $Ncb5or^{-/-}$ (B) mice or after 7 days on HF (D,F: WT, E,G: $Ncb5or^{-/-}$) and from 6-week-old SC-fed $Ncb5or^{-/-}$ mice (C). All β -cells are delineated with dashed lines, and those from $Ncb5or^{-/-}$ mice contained dER (B, C and D). Two adjacent α -cell are marked as “ α ” (B). Two adjacent β -cells from 6-week-old $Ncb5or^{-/-}$ mice displayed minor and severe dER (C, left and right, respectively). Key subcellular organelles are labeled: rER: rough endoplasmic reticulum; dER: distended ER; Golgi: Golgi apparatus; mito: mitochondria; SG: secretory granules (for insulin); N: nucleus; *: visible ribosomes. A, B, C,D, and E: $\times 2900$; F and G: $\times 19000$.

FIG. 5. Abundance of dER-containing β -cells and islet β -cell content of SC- and HF-fed Ncb5or^{-/-} mice

(A) The percentage of dER-containing β -cells among total β -cell population of SC-fed Ncb5or^{-/-} mice at age 5 (open bars) or 6 weeks (grey bars) and of HF-fed mice at age 5 weeks (closed bars). (B) Islet β -cell content of Ncb5or^{-/-} and WT mice at the above ages. The cell-content in individual islets was determined by counting β -cells with distinct TEM features. Statistical significances are shown with asterisks over the representative graph bars. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$.

Table 1

Islet lipid content and fatty acid desaturation indices of Ncb5or^{-/-} and WT mice at age 4.5 weeks after consumption of a HF diet for 3 days.

	FFA			TAG			PL	
	WT	Ncb5or ^{-/-}	p	WT	Ncb5or ^{-/-}	p	WT	Ncb5or ^{-/-}
Total [nmole/mg]	197±23	280±27	0.04	36.4±6.4	70.6±10.6	0.02	1280±100	1440±220
SFA [nmole/mg]	144±16	218±20	0.02	26.8±5.4	48.3±5.8	0.03	522±42	605±82
MUFA [nmole/mg]	37.9±8.6	41.4±8.8	NS	5.4±0.9	13.6±3.2	0.02	213±18	230±34
C16:0 [nmole/mg]	69.8±8.2	97.8±7.8	0.04	10.8±2.1	20.7±2.7	0.02	226±21	267±31
C18:0 [nmole/mg]	49.0±5.9	75.6±8.1	0.02	7.8±1.9	13.2±0.9	0.06 (NS)	259±19	284±42
C16:1 [nmole/mg]	3.2±0.6	4.8±1.0	NS	0.47±0.15	1.24±0.51	NS	8.7±1.0	10.2±1.7
C18:1 [nmole/mg]	32.7±7.8	32.6±7.7	NS	3.6±0.5	9.1±1.6	0.004	183±15	204±29
DI (C16)	0.046±0.007	0.050±0.011	NS	0.041±0.006	0.063±0.020	NS	0.039±0.002	0.038±0.003
DI (C18)	0.68±0.16	0.43±0.09	NS	0.52±0.06	0.69±0.12	NS	0.71±0.03	0.73±0.02

Total amounts of fatty acids [nmol/mg proteins] and desaturation indices (DI) of 16-carbon (C16) and 18-carbon (C18) fatty acid species are displayed for intracellular pools of free fatty acids (FFA), triacylglycerol (TAG) and phospholipid (PL) substituents. Mean values ± S.E. are displayed, and tabulated *p* values pertain to statistical comparisons between WT (n=5-6) and Ncb5or^{-/-} (n=4-5) mice. "NS" denotes a comparison that is not significant statistically, characterized by a *p* value exceeding 0.05.

Table 2

Islet transcript levels for genes involved in insulin and glucagon biosynthesis, ER stress responses, or fatty acid metabolism.

Pathway	Gene	SC			HF		
		WT	Ncb5or ^{-/-}	Ncb5or ^{-/-} / WT Fold Change	WT	Ncb5or ^{-/-}	Ncb5or ^{-/-} / WT Fold Change
Endocrine synthesis	Insulin	250000±4000	260000±40000	1.0	200000±30000	180000±30000	0.9
	Gln	50000±11000	110000±11000 ^a	2.2	31000±6000	76000±13000 ^a	2.5
ER stress	Xbplt	85±10	96±9	1.1	73±8	93±17	1.3
	Xbpls	55±9	63±6	1.1	39±4	37±6 ^b	1.0
	ATF6α	49±8	72±5 ^a	1.5	33±4 ^b	49±5 ^{a,b}	1.5
	BIP	199±25	179±20	0.9	146±21	155±24	1.1
	Grp94	479±59	442±44	0.9	446±61	461±41	1.0
	ATF3	31±12	40±24	1.3	34±8	68±21 ^a	2.0
	CHOP	41±12	56±14	1.4	76±24	139±37 ^c	1.8
Fatty acid metabolism	PGC-1α	1.2±0.2	3.9±0.5 ^a	3.2	1.2±0.4	3.7±1.7 ^a	3.0
	FAS	75±21	115±19	1.5	64±16	93±24	1.5
	SCD1	185±32	261±27	1.4	101±15 ^b	106±16 ^b	1.1
	SCD2	92±16	160±25 ^a	1.7	66±7	75±16 ^b	1.1
	CD36	27±8	41±12	1.5	25±8	50±11	2.0
	LPL	48±6	77±11 ^a	1.6	31±6 ^b	34±6 ^b	1.1

Tabulated values pertain to WT or Ncb5or^{-/-} mice fed SC or 3 days of a HF diet and were determined by qRT-PCR. Signal for each individual transcript was normalized to that for 18s rRNA. Mean values ± S.E. are displayed, and tabulated *p* values pertain to statistical comparisons between WT (n = 6) and Ncb5or^{-/-} (n = 5) mice fed SC or HF diets. Abbreviations include: Ins2, insulin gene 2; Gln, Glucagon; ATF6α and ATF3, activating transcription factor 6α and 3 respectively; Xbpls, X-box binding protein spliced form; CHOP, CCAAT/enhancer-binding protein homologous protein; PGC1α, peroxisome proliferator-activated receptor γ-coactivator; SCD1,2, stearoyl-CoA desaturase 1 and 2, respectively; FAS, fatty acid synthase; LPL, lipoprotein lipase; CD36, a membrane glycoprotein in fatty acid transport. A statistical significance is characterized by a *p* value not exceeding 0.05.

^a significantly different between Ncb5or^{-/-} and WT on the same diet,

^b significantly lower under HF than SC of the same genotype,

^c significantly higher under HF than SC of the same genotype.