Targeted ablation of the novel flavoheme reductase Ncb5or knock-out (KO) results in progressive loss of pancreatic β-cells and white adipose tissue over time. Lipoatrophy persisted in KO animals in which the confounding metabolic effects of diabetes were eliminated by islet transplantation (transplanted knock-out (TKO)). Lipid profiles in livers prepared from TKO animals were markedly deficient in triglycerides and diacylglycerides. Despite enhanced expression of stearoyl-CoA desaturase-1, levels of palmitoleic and oleic acids (Δ9 fatty acid desaturation) were decreased in TKO relative to wild type controls. Treatment of KO hepatocytes with palmitic acid reduced cell viability and increased apoptosis, a response blunted by co-incubation with oleic acid. The results presented here support the hypothesis that Ncb5or supplies electrons for fatty acid desaturation, offer new insight into the regulation of a crucial step in fatty acid biosynthesis, and provide a plausible explanation for both the diabetic and the lipoatrophic phenotype in Ncb5or−/− mice.

The most prevalent and life-threatening metabolic disorders, type 2 diabetes and obesity, arise from the complex interplay of a large number of genetic determinants. Characterizing polygenic disorders is challenging, requiring insight into plausible candidate genes and analysis of genomic data on numerous kindred. Mouse models offer an attractive independent source of information on genes important in pancreatic β-cell and adipocyte viability and homeostasis. We have been investigating a novel flavoheme reductase that plays an important role in pancreatic β-cell viability and maintenance of white adipose tissue. Ncb5or (alternatively called b5 + b5R and cyb5r4) is a single 58-kDa polypeptide composed of two well-characterized domains tethered by a unique 90-residue hinge (1). The 130-residue N-terminal domain bears strong homology to classic microsomal cytochrome b5, a six-coordinate heme protein. At the C terminus, there is a 300-residue domain with strong homology to classic microsomal cytochrome b5 reductase, a flavoprotein. Ncb5or is of special interest because it is highly conserved in a wide range of animals from mammals to flies and worms and because there is no other example in the animal kingdom of such a two-domain protein. Ncb5or is expressed in a wide variety of organs, tissues, and cell lines and localized in the endoplasmic reticulum (2). Because of its high negative redox potential, Ncb5or would be expected to function primarily as an electron donor.

Mice with a global knock-out of Ncb5or have normal embryonic and fetal development and normal viability at birth with no gross anatomic abnormalities (3). However, after the first month of life, Ncb5or−/− mice develop diabetes because of the loss of pancreatic β-cells. In addition, there is progressive atrophy of white adipose tissue (WAT). The diabetic and lipoatrophic phenotype of the Ncb5or−/− mouse raises the question of how this novel reductase impacts β-cell and adipocyte viability and function.

“Classic” cytochrome b5 (b5) and cytochrome b5 reductase (b5R) are encoded by separate genes and are each expressed as two mRNA isoforms distinguished by alternative splicing (b5)(4, 5) along with alternative promoter usage (b5R) (6–8). Soluble forms of b5 and b5R are expressed specifically in erythroid cells, where they function in tandem as electron donors for reduction of methemoglobin. In contrast, microsome-bound b5 and b5R are expressed in a wide range of cells and fulfill a number of metabolic functions including the supply of electrons for fatty acid desaturation (9–12). In the Δ9 desaturation reaction, stearoyl CoA desaturase (SCD) catalyzes the conversion of C16 palmitic acid to palmitoleic acid and C18 stearic acid to oleic acid (11). The oxidative formation of the double bond requires the presence of oxygen and electron transport: NADH → b5R → b5 → SCD. Other fatty acid desaturases, Δ5 and Δ6, contain a b5-like domain at the N terminus of their respective polypeptides (13–15) and therefore require only NADH and b5R for electron transfer (16).
Because of the presence of b5 and b5R domains within Ncb5or, this reductase might serve as an alternate or surrogate source of electrons for SCD. In this report, we further characterize Ncb5or null mice with islet transplantation and present evidence that Ncb5or deficiency results in impaired Δ9 desaturatation. Moreover, we show that primary hepatocytes from Ncb5or null mice have enhanced sensitivity to the toxic effects of palmitate. These results provide a plausible explanation for both the diabetic and the lipoatrophic phenotype in Ncb5or−/− mice and provide new insights into the regulation of a crucial step in fatty acid biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Palmitate acid and oleic acid were purchased from Sigma (St Louis, MO). 5.0 mM FFA (free fatty acid) stock solutions were prepared as described by Roche et al. (17). The sodium salt of the corresponding fatty acid was dissolved in Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 2.5 mM CaCl2·2H2O, pH 7.4) containing 5% fatty acid-free bovine serum albumin (BSA) with shaking at 37 °C for 8 h under nitrogen atmosphere to prevent oxidation (molar ratio of fatty acid to BSA ~6). The pH of the fatty acid solution was then adjusted to 7.4 and filtered through a 0.2-μm filter. The final concentration of fatty acid was measured using a non-esterified fatty acid C kit (Wako Chemicals, Richmond, VA), and adjusted to 5 mM. Finally, the prepared stock solutions were stored in liquid nitrogen to prevent oxidation (17).

All procedures involving animals were approved by the Animal Care and Use Committee of Brigham and Women’s Hospital and Children’s Hospital Boston. Mice were housed in a pathogen-free barrier facility on a 12-h light/12-h dark cycle on a standard corn oil-based rodent chow, 3.6 kcal/g (Prolab Iso-pro RMH 3000 5P76).

**Islet Transplantation**—A sustained release insulin implant (Linbit; LinShin, Inc.) was placed subcutaneously under the mid-dorsal skin of Ncb5or−/− mice in the prediabetic stage (<4 weeks of age). The implant provided a delayed, sustained release of insulin to prevent hyperglycemia until transplantation at 6 weeks. Islets were prepared from Ncb5or−/− mice, as described previously (3), for transplant into syngeneic (inbred) Ncb5or−/− mice.

Islets (minimum 300 islets/recipient) were pelleted in small gauge PE50 tubing for transplant and injected beneath the left kidney capsule of Linbit-implanted, 6-week-old Ncb5or−/− mice. Fasting blood glucose was used to monitor whether transplanted Ncb5or−/− mice were cured of diabetes. Glucose tolerance tests (GTT) were performed prior to sacrifice to confirm that mice were not diabetic and responded physiologically to a glucose load. Litter-matched Ncb5or−/− mice underwent a sham operation.

**Tissue Preparation and Analysis**—Liver and other specimens were derived from mice sacrificed after overnight fast. Tissue for analysis was fixed in either buffered 4% paraformaldehyde solution or Bouin’s solution, embedded in paraffin, and sectioned for analysis. Slides were stained with hematoxylin and eosin (H&E) for islet identification and analysis. For cell size measurements, epididymal white adipose tissue prepared from 6-week-old mice was examined microscopically (Nikon) using bright field exposure in conjunction with the Metamorph program (Molecular Devices). Cells were scored in a blinded manner for n = 5 age/litter-matched mice representing each genotype, and results are reported as mean ± S.E.

**Blood and Tissue Analyses**—All specimens except for GTT were collected after overnight fast. Tissue samples were flash-frozen in liquid nitrogen and stored at −80 °C until used for analyses.

Blood glucose concentration was measured using the “One Touch” blood glucose monitoring system (Lifescan). Intraportal GTT were performed as described previously (3). Retro-orbital blood collection was performed on anesthetized mice prior to sacrifice. Kits were used to measure leptin (mouse leptin enzyme-linked immunosorbent assay; Crystal Chem Inc.) and ketones (Precision Xtra ketone strips) in serum.

**Body Fat Composition**—Mice were fasted for 4 h and anesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg; intraperitoneal, single injection). Body fat composition was analyzed by dual energy x-ray absorptiometry (DEXA) with a PIXImus scanner (GE Healthcare-Lunar).

**Isolation of Mouse Adipocytes**—Epididymal white adipose tissue was rapidly dissected out, placed in a Petri dish, and washed with phosphate-buffered saline solution. Tissue was transferred into a vial containing 1.0 ml of collagenase digestion solution (1 mg/ml type II collagenase (Sigma); 25 mM HEPES, 3.5% BSA, 2 mM glucose) in Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 2.5 mM CaCl2·2H2O, pH 7.4). Tissue was minced in the collagenase solution, incubated at 37 °C for 30 min, and washed in Krebs-Ringer buffer (25 mM HEPES, 1% BSA, 2 mM glucose, pH 7.4). Adipocytes were then gravity-filtered through gauze, resuspended in Dulbecco’s modified Eagle’s medium, and examined using a Nikon Eclipse TE300 microscope.

**Isolation of Mouse Hepatocytes**—Mouse hepatocytes were isolated from 3–4-week-old wild type and Ncb5or−/− male animals by the two-step perfusion procedure using Perfusion Solution I (0.5 mM EGTA, and 5.5 mM glucose in Hanks’ balanced salt solution.) and Perfusion Solution II (1.5 mM CaCl2, 5.5 mM glucose, with 0.0375% collagenase in Hanks’ balanced salt solution.), separately, as described by Kim et al. (18). Isolated hepatocytes were cultured in Williams’ medium E (Invitrogen) containing penicillin/streptomycin and glutamine. After isolation, hepatocytes were incubated at 37 °C in a humidified atmosphere (5% CO2, 95% air) in Williams’ medium E medium containing penicillin/streptomycin and glutamine. After a 3-h incubation, cells were treated in Williams’ medium E medium containing fatty acid-free BSA only (control condition), 0.5 or 1 mL palmitic acid, 0.5 or 1 mL oleic acid, or the combination of 0.5 mL palmitic acid and 0.5 mL oleic acid. Fatty acid-free BSA was added to the medium and adjusted to a final concentration of 1%. After the treatment, cells were collected at each time point (1, 2, or 4 h) and subjected to analysis of viability with trypan blue and apoptosis with TUNEL staining.

**Tissue Lipid Analyses**—Liver was freshly harvested from wild type (WT) and TKO animals and flash-frozen in liquid nitrogen. Tissue lipid analysis was performed by Lipomics Technologies, Inc. (West Sacramento, CA). Briefly, lipids were extracted using chloroform:methanol (2:1 v/v), and individual
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lipid classes were separated by liquid chromatography. Each lipid class was transesterified in 1% sulfuric acid in methanol under a nitrogen atmosphere at 100 °C for 45 min. Fatty acid methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene. Fatty acid methyl esters were separated and quantified by capillary gas chromatography (Agilent Technologies model 6890).

Microarrays—Samples were prepared from the livers of three pairs of 12-week-old male, litter-matched TKO and WT mice. Total RNA was prepared from liver using the Qiagen mini-RNA isolation kit with quality and concentration determined by Bioanalyzer quality control analysis. Hybridization of samples prepared from WT and KO animals at various ages and presented as two separate time intervals (inset). C, gonadal fat prepared from WT and KO animals at 6.5 weeks of age. D, adipocytes prepared from gonadal fat pads in C.E, adipocyte cell area (left panel; n = 5 animals, 6–7 weeks of age, for each genotype, >20 cells per n) and representative H&E sections (right panel). Statistical significance: a, p < 0.025.

FIGURE 1. Loss of WAT in Ncb5or−/− mice. A, body weight (top panel) and total body fat measured via DEXA scan (bottom panel) of Ncb5or WT (open symbol) and KO (filled symbol) mice over time. Each data point represents n = 7–8 animals. A single cohort of mice was used to prepare both the top and the bottom panels with data collected simultaneously; values are significantly different starting at 6 weeks, p < 0.001. At each time point, B, body weight (top panel) and total body fat measured via DEXA scan (bottom panel) of Ncb5or WT (open symbol) and KO (filled symbol) mice over time. Each data point represents n = 7–8 animals. A single cohort of mice was used to prepare both the top and the bottom panels with data collected simultaneously; values are significantly different starting at 6 weeks, p < 0.001.

RESULTS

Characterization of the Lipoatrophy Phenotype—Our initial description of the Ncb5or−/− mouse included a detailed account of the progressive loss of pancreatic β-cells beginning at 4 weeks of age (3). We also reported concomitant loss of white adipose tissue despite increased food intake but could not state whether it was an independent feature of Ncb5or deficiency or merely due to increased hypermetabolism and catabolism that accompanies frank diabetes. Therefore, we have investigated the lipoatrophy in Ncb5or−/− mice in more detail. As shown in Fig. 1A, top panel, the body weight of knock-out (KO) mice fed standard rodent chow over 5 weeks of age lagged behind that of the WT animal, in which there was the expected gradual weight gain over the next 10 weeks. This weight gain in WT mice could be accounted for by a comparable increase in total body fat as assessed by DEXA scanning (Fig. 1A, bottom panel). In contrast, DEXA scans of the Ncb5or−/− mice revealed a constant low level of total body fat that was just at the threshold of detection. In Ncb5or−/− mice, we observed progressive loss of all visible deposits of white adipose tissue, as shown by a plot of the mass of perigonadal white fat (Fig. 1B). Fat pads prepared from Ncb5or−/− animals aged 6.5 weeks underwent a marked reduction in size (Fig. 1C), and adipocytes dispersed from these fat pads were significantly smaller in the KO animal (Fig. 1D). A more detailed examination of adipocytes in H&E sections prepared from these fat pads revealed that the KO cells were significantly smaller (693 ± 29 ± 57 μm2 S.E.) than WT cells (1120 ± 91 μm2) (Fig. 1E). Thus the reduction in mass of white adipose tissue in KO mice appears to be due at least in part to a marked decrease in cell size. In contrast to white adipose tissue, brown adipose tissue prepared from the same WT and KO animals showed no apparent abnormality in morphology or size (results not shown).

Islet-transplanted Ncb5or−/− Mice—To eliminate the confounding metabolic effects of diabetes, islet transplants were performed on KO BALB/c animals to prevent hyperglycemia and correct the diabetes. These transplanted knock-out animals are designated TKO. A sham operation was performed on uncorrected KO littermates (UKO).

Fasting blood glucose levels from 12-week-old WT and TKO animals were not significantly different, whereas the UKO ani-
mals were significantly higher (Fig. 2B). Both WT and TKO animals were able to clear glucose efficiently as assessed by GTT, with curves that were indistinguishable, whereas the UKO animal, as expected, had markedly impaired glucose tolerance (Fig. 2A). Pancreatic sections (Fig. 2D) revealed, as expected, round and healthy WT islets, whereas the endogenous TKO islets were misshapen in appearance, similar to KO islets examined previously (3). In contrast, the islet tissue transplanted under the kidney capsule appeared healthy. The transplanted islets were in the KO mice for at least 6 weeks (the length of time that results in the loss of KO islets). Nevertheless, they remained fully functional. Thus the transplanted islets maintained normal blood glucose but did not protect the endogenous islet, suggesting that the defect leading to islet loss is intrinsic and that Ncb5or plays a key role in beta cell maintenance.

In TKO animals, body weight continued to decrease although diabetes had been corrected. At age 12 weeks, mean body weights were 24.4±1.1 g for WT mice, 14.2±1.1 g for KO mice, and 16.2±0.8 g for TKO mice. Overnight fasted serum samples from WT, TKO, and UKO animals were tested for ketones and leptin. Ketone levels did not demonstrate a discernable trend, and no significant differences were observed. Leptin levels were normal in WT
animals but could not be detected in TKO and UKO animals (Fig. 2C), consistent with a marked reduction in white adipose tissue. As expected, a large epididymal fat pad was present in all WT animals examined, whereas the fat pad was markedly smaller in TKO animals (Fig. 2E). Moreover, the TKO mice had comparable diminution in perinephric and mesenteric white adipose tissue (Fig. 2E, right). These results in the TKO mice show that the lipoatrophy observed in Ncb5or−/− mice is a result of the enzyme deficiency per se and not a consequence of metabolic perturbations induced by diabetes.

**Lipid Analyses in TKO Mice**—Since Ncb5or contains b5 and b5R domains that could be an alternate source of electrons for SCD, we next asked whether the TKO mouse had a derangement in fatty acid desaturation. As shown in Fig. 3A, when compared with wild type, there was a marked increase in stearoyl CoA desaturase-1 (SCD1) transcription in livers of TKO mice. Western blots showed that prediabetic Ncb5or−/− mice also had enhanced expression of SCD1 protein (Fig. 3B). In contrast, TKO and WT mice had no significant differences in the liver expression of SCD-2 or SCD-3 (Fig. 3A). Moreover, there were no significant differences in expression of cytochrome b5 mRNA (supplemental Table 1) and protein (Fig. 3B) as well as b5R mRNA (supplemental Table 1). Lack of an effective antibody against b5R precluded assessment of protein levels by Western blotting.

We obtained lipid profiles in livers prepared from WT and TKO animals. Liver was chosen for study over white adipose tissue because time-dependent involution of the latter limited adequate sampling in older animals and confounded normalization of results. Importantly, Ncb5or−/− mice have normal liver histology and normal serum liver function tests (3). As shown in Fig. 3C, the TKO liver samples were markedly deficient in triglycerides (TAG) and diacylglycerides and moderately deficient in free fatty acids (FFA), whereas the other classes of liver lipids were normal. As shown in Fig. 3D, levels of the monounsaturated fatty acids in the n-7 and n-9 pathways (16:1n-7 and 18:1n-9, respectively) were decreased in TAG from TKO liver. When these values are plotted as nmol FFA/nmol TAG ×100 (mol %), the same pattern is observed (results not shown). Plots of the ratio of the monounsaturated fatty acids (16:1 and 18:1) to total liver lipid relative to their saturated counterparts (16:0 and 18:0) are shown in Fig. 4, A and B. The desaturation index is calculated by dividing the monounsaturated fatty acid by the saturated form. Following examination of specific lipid species, both the C16 and the C18 desaturation indices were lower in TKO versus wild type in TAG, cholesterol ester (CE), diacylglycerides (DAG), and FFA (Fig. 4, C and D), indicating that the absence of Ncb5or expression impairs C16 and C18 desaturation of palmitate and stearate in a number of classes of lipids. Both pathways are catalyzed by the Δ9 desaturase. The results presented in Fig. 4 strongly suggest that Ncb5or is involved in Δ9 fatty acid desaturation, providing electrons to the SCD1 desaturase, and that Ncb5or−/− mice have decreased ratios of 16:1/16:0 and 18:1/18:0. A full profile of all lipid classes from livers of TKO and WT mice is included as a spreadsheet (supplemental Table 2).

Microarray analysis was performed on mRNA from livers of TKO and WT mice. As shown in supplemental Table 1, genes important in lipid metabolism were expressed at about the same level in the two groups. One gene encoding for PGC-1α was markedly (5-fold) increased in TKO livers, and none were comparably down-regulated. PGC-1α, a PPARγ coactivator, coordinates regulates metabolic pathways in a tissue-specific manner and is a potent stimulator of mitochondrial biogenesis (19, 20). PGC-1α is required for the induction of a number of antioxidant enzymes (21). However, the expression of these antioxidant enzymes was not significantly altered in mRNA from liver or islets of Ncb5or−/− mice (22).

**Fatty Acid Toxicity in Ncb5or−/− Hepatocytes**—As detailed under “Discussion” below, both C16 and C18 (palmitate and stearate) are toxic to β-cells (23–25) and a variety of other cells (26–29) including liver (30). This toxicity can be partially or fully prevented by co-incubation with monounsaturated oleic acid. Despite repeated attempts, we were not able to carry out experiments on the most relevant cells, isolated islets, and adipocytes because of the severity of the KO phenotype, enhanced fragility of these cells, and low preparative yields. Therefore, we used primary hepatocytes, prepared by collagenase treatment of livers of 3.5-week-old wild type and Ncb5or−/− mice. At this age, the animals are normoglycemic (3). As shown in Fig. 5, exposure to the saturated fatty acid palmitic acid (0.5 mM) resulted in decreased cell viability (measured by trypan blue exclusion) and enhanced TUNEL staining. Palmitate was chosen because it is the most abundant fatty acid in the circulation (31). In contrast, exposure to the monounsaturated oleic acid (0.5 mM) had no effect on either viability or TUNEL stain-
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**DISCUSSION**

Our central challenges are to identify the primary biologic role of Ncb5or and to understand the molecular basis for the diabetic and lipoatrophic phenotypes in the Ncb5or−/− mouse. Our islet transplantation experiments (Fig. 2) show that the progressive loss of WAT is a primary result of absent Ncb5or expression rather than secondary to hypercatabolism of severe diabetes.

The most plausible function for Ncb5or is supplying electrons for fatty acid desaturation. As mentioned in the Introduction, the N-terminal and C-terminal domains of Ncb5or bear strong homology with classic cytochrome b₅ and cytochrome b₉ reductase, which supply electrons to stearoyl CoA desaturase (SCD). Ncb5or−/− mice appear to have an impairment in the Δ9 pathway of fatty acid desaturation, which is mediated by SCD. As shown in Fig. 4, triglyceride and other classes of lipid in the livers of Ncb5or−/− mice have marked reductions in the ratios of oleate to stearate and of palmitoleate to palmitate. These analyses were performed on islet-transplanted mice, and therefore the results were not influenced by confounding metabolic effects of diabetes. The observed impairment in the Δ9 pathway in Ncb5or−/− mice is particularly striking considering the marked increase in hepatic SCD-1 expression (Fig. 3, A and B).

We also found, as shown in Fig. 5, that primary hepatocytes from Ncb5or−/− mice are unusually sensitive to the cytotoxic effects of palmitate, but not oleate, or oleate plus palmitate. The fatty acids in these experiments were bound to albumin at ratios that are expected to produce nanomolar free fatty acid concentrations, roughly within the range encountered in vivo (28, 32). Although these experiments are inherently artificial, the increased sensitivity of Ncb5or−/− hepatocytes to palmitate is likely due to defective conversion to non-toxic monounsaturated palmitoleate and oleate.

Defective fatty acid desaturation is likely to be an important contributor to both the diabetic and the lipoatrophic phenotype in Ncb5or−/− mice. Pancreatic β-cells are particularly susceptible to the toxicity of saturated fatty acids. In vitro, saturated FFA induce β-cell toxicity.

**FIGURE 5. Fatty acid toxicity on WT and KO primary hepatocytes.** Primary hepatocytes from three 3.5-week-old Ncb5or−/− and three wild-type mice were incubated with 0.5 or 1 mM concentrations of palmitate (P) or oleate (O) or the combination (C) (0.5 mM each) in buffer containing 1% bovine serum albumin for 1 or 4 h. Control: buffer with 1% BSA. A, cell viability was measured using a trypan blue exclusion assay. Statistical significance: *p* < 0.05. B, TUNEL staining was used to assess apoptosis.
more likely than necrosis as a cause of cell loss since H&E sections revealed no evidence of inflammation. Saturated FFA triglycerides in the liver were markedly increased in Ncb5or+/−/− animals (Fig. 3, A and B).

It is unlikely that Ncb5or is the sole provider of electrons for conversion of stearate to oleate. Lipid analyses on a patient with congenital methemoglobinemia due to generalized deficiency of cytochrome b5 reductase revealed no gross defect in C16:1/C16:0 and C18:1/C18:0 ratios (50–52). These results suggest that there are alternate sources of electrons for Δ9 desaturation and that Ncb5or may provide this function.

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