Neurogenin 3-Specific Dipeptidyl Peptidase-2 Deficiency Causes Impaired Glucose Tolerance, Insulin Resistance, and Visceral Obesity

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The control of glucose metabolism is a complex process, and dysregulation at any level can cause impaired glucose tolerance and insulin resistance. These two defects are well-known characteristics associated with obesity and onset of type 2 diabetes. Here we introduce the N-terminal dipeptidase, DPP2, as a novel regulator of the glucose metabolism. We generated mice with a neurogenin 3 (NGN3)-specific DPP2 knockdown (kd) to explore a possible role of DPP2 in maintaining metabolic homeostasis. These mice spontaneously developed hyperinsulinemia, glucose intolerance, and insulin resistance by 4 months of age. In addition, we observed an increase in food intake in DPP2 kd mice, which was associated with a significant increase in adipose tissue mass and enhanced liver steatosis but no difference in body weight. In accordance with these findings, the mutant mice had a higher rate of respiratory exchange than the control littermates. This phenotype was exacerbated with age and when challenged with a high-fat diet. We report, for the first time, that DPP2 enzyme activity is essential for preventing hyperinsulinemia and maintaining glucose homeostasis. Interestingly, the phenotype of NGN3-DPP2 kd mice is opposite that of DPP4 knockout mice with regard to glucose metabolism, namely the former have normal glucagon-like peptide 1 levels but present with glucose intolerance, whereas the latter have increased glucagon-like peptide 1, which is accompanied by augmented glucose tolerance. (Endocrinology 150: 5240–5248, 2009)
specific DPP4 inhibitor approved by the Food and Drug Administration for this purpose (5).

DPP2 is a lesser known, nevertheless important, member of the DPP subfamily of serine proteases (9). This enzyme forms homodimers, similar to DPP4. However, unlike DPP4, which is an ectoenzyme expressed on the surface of many cell types and shed in active form into the plasma, DPP2 is localized to the vesicular compartment of the cytosol (10). Furthermore, DPP2 bears no significant nucleotide or amino acid homology with DPP4 (9). Whereas the order of the catalytic amino acids, Ser-Asp-His, is identical in DPP4 and DPP2, the spacing of the catalytic triad is dissimilar, suggesting different substrate fine specificities (11). Nevertheless, DPP2 and DPP4 share preference for Ala and/or Pro in the P1 position; thus, enzymatic action of both enzymes on the same peptides/proteins is possible. Although the DPP2 recognition sequence is well defined (12), an apparent peptide size limitation in the in vitro protease assay has prevented the definition of its endogenous substrate(s) so far (Huber, B. T., unpublished observation).

These data suggest that a cofactor is needed to define real activity and the relevant substrate(s) of DPP2. In particular, it is unknown whether peptide neurotransmitters, which have the predicted N-terminal cleavage site and are involved in metabolic regulation, such as neuropeptide Y and peptide YY (13, 14), are affected in vivo by DPP2. Knockout (ko) of DPP2 and constitutive DPP2 knockdown (kd) are embryonic lethal (Huber, B. T., unpublished observation). Therefore, to explore a potential physiological role of DPP2 in vivo, we used the conditional kd approach. Specifically, we generated neurogenin 3 (NGN3)-DPP2 kd mice because NGN3 is expressed in all precursors of the enteroendocrine cells and in the pancreas as well as discrete regions of the hypothalamus and brain stem (15). We hypothesized that these mice would exhibit enhanced glucose tolerance and insulin sensitivity, similar to what has been observed in DPP4 ko mice (16, 17) due to enzymatic action of DPP2 on GLP-1. Intriguingly however, the phenotype of NGN3-DPP2 kd mice was opposite of the DPP4 ko mice, namely they had unaltered levels of active GLP-1 but presented with hyperinsulinemia, accompanied by impaired glucose tolerance, insulin resistance, enhanced liver steatosis, and visceral obesity. The significance of these novel results in terms of treatment strategies for type 2 diabetes is discussed.

Materials and Methods

Generation of conditional DPP2 kd mice

Numerous short hairpin (sh) RNAs against mDPP2 were designed, using the pSico Oligomaker 1.5 program, available on the Tyler Jacks Lab web site (http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html), and tested for their capacity to kd murine DPP2 protein and enzyme activity. The optimal sequence that suppressed DPP2 expression and activity by 90% (D. A. Mele, Oakes, B., Huber, B. T., manuscript in preparation) was subcloned into the lentiviral construct, pSico (18). This vector allows conditional expression of shRNA by Cre-loxP regulation. The shRNA in pSico is not transcribed until a Cre-mediated recombination occurs, which is marked by the loss of enhanced green fluorescent protein (GFP) and expression of shRNA (18) (Fig. 1A). Using this shRNA sequence in the conditional pSico vector, we infected ES cells and injected them into the blastocysts of pregnant mice, and several male chimeric founder mice were obtained that differed in GFP expression (Mele et al., in preparation). The two male mice with highest GFP expression were chosen to mate with transgenic (tg) mice that express Cre in a tissue-specific manner. The NGN3-Cre tg mouse has been previously described (15). Crossing conditional DPP2 kd mice with NGN3-Cre tg mice resulted in similar numbers of Cre + offspring that were positive/negative for expression of the shDPP2 kd construct, indicating that NGN3-DPP2 kd does not affect viability. The littermates, NGN3-Cre and shDPP2-loxP, served as controls in all experiments. Only male mice were used in the presented work. The shDPP2-loxP mice were generated from 129/J ES cells (19), implanted into FVB mice to generate chimeric mice. These mice have been backcrossed to C57BL/6 for four generations, using the speed congenic approach (20). The NGN3-Cre mice were on the CD1 background.

GFP immunofluorescence

GFP-loxP mice were crossed with NGN3-Cre tg mice, leading to NGN3-specific GFP expression. NGN3-Cre and GFP-loxP littermates served as negative control. These mice were anesthetized with pentobarbital (50 mg/kg ip) and perfused transcardially with 10 ml of 0.01 M PBS (pH 7.4), followed by 40 ml 4% paraformaldehyde in phosphate buffer (pH 7.4). Brain, small intestine, liver, and pancreas were dissected out, and a block containing hypothalamus was isolated from the brain using mouse brain matrix. All tissues were postfixed in the same fixative and immersed in 25% sucrose solution in PBS overnight. After freezing the brain and other tissues on powdered dry ice, the hypothalamus was sectioned on a cryostat (CM3050S; Leica, Heidelberg, Germany) to obtain three sets of serial, coronal sections. Sections were collected in PBS and stored at −20 C in cryoprotectant solution. One set of sections was processed for GFP immunofluorescence. Sections were washed in PBS, pre-treated with 0.5% Triton X-100 in PBS for 20 min, followed by incubation in 10% normal horse serum in PBS for 30 min. They were then incubated in rabbit polyclonal GFP antiserum (In-vitrogen, Carlsbad, CA; catalog no. A6455), diluted 1:5000 in antibody diluent (1% normal horse serum, 0.2% Photo Flo; Kodak, Rochester, NY; 0.08% sodium azide) for 2 d at 4 C, followed by incubation in Cy3-conjugated donkey antirabbit IgG (1:250; Jackson ImmunoResearch, West Grove, PA) overnight at 4 C. After washing in PBS, the sections were mounted on Superfrost slides (Fisher Scientific, Fair Lawn, NJ), coverslipped with Vectashield mounting medium with 4’,6’-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA) and observed under Axiosplan 2 epifluorescence microscope (excitation 540–590 nm, bandpass 595 nm, and emission 600–660 nm; Zeiss, New York, NY). Images were captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) and ad-
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FIG. 1. Production of NGN3-DPP2 kd mice. A, Conditional vector used to express shRNA for lentiviral infection of embryonic stem cells. shRNA in pSico is not transcribed until a Cre-mediated recombination occurs, which is marked by the loss of enhanced GFP and expression of shRNA (adapted from Ref. 4). B, NGN3-GFP expression profile in brain. Frozen sections of the hypothalamus were immunolabeled, using anti-GFP antiserum. A high concentration of GFP+ cells is seen in the hypothalamic VMN, with smaller populations in the hypothalamic ARC. The organization of the ARC and VMN is shown with a dashed line (1). Arrows (1, 2) indicate GFP+ cells of the VMN; arrowheads (1, 2) indicate GFP+ cells in the ARC. Fibers are present in the hypothalamic paraventricular nucleus (PVN) (3). No immunolabeling is seen in the hypothalamus of NGN3-Cre− GFP-loxP mice (4). III, Third ventricle; DMN, dorsomedial nucleus; ME, median eminence. Scale bar, 200 μm (1) and 100 μm (2–4). C, NGN3-GFP expression profile in different tissues. Frozen sections of small intestine (1, 2), pancreas (3, 4), and liver (5, 6) were immunolabeled, using FITC-conjugated anti-GFP antiserum (1, 3, and 5, phase contrast; 2, 4, and 6, immunofluorescence). D, DPP2 mRNA is specifically kd in the liver of NGN3-DPP2 kd mice (n = 2) and control mice (n = 2, NGN3-Cre, shDPP2-loxP), using the RNaseq minikit per the manufacturer’s instructions (QIAGEN, Valencia, CA). Deoxyribonuclease-treated (TURBO DNA-free; Ambion, Austin, TX) total RNA (1 μg) was reverse transcribed with the iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA). Taqman qRT-PCR to quantify DPP2 mRNA expression was performed (probe: CTGAGCAC-CGGTACTATG; forward primer: GGAGGCGCT-GCTTGCTCTT; reverse primer: CACCGAAAG-GAACCGATTTC) on an ABI 7300 system (Applied Biosystems, Foster City, CA). The 18S VIC Taqman probe was used as internal control for all reactions.

Sudan Black B staining

Frozen sections of livers were fixed in 10% formalin, washed twice in water and propylene glycol, and incubated with Sudan Black. The sections were then washed with 85% propylene glycol, rinsed in distilled water, and mounted with mounting media. The percentage areas of Sudan Black stain were quantified, using image analytical software MetaXpress 2.0 (Molecular Devices Analytical Technologies, Ottawa, Ontario, Canada).

GLP-1 bioassay

Tail blood was collected on ice from NGN3-DPP2 kd and control littermates in the morning after an 18-h fast and 20 min after oral glucose challenge, and a standard nonspecific DPP inhibitor, Valboroph-4: Pro (10 μM final concentration), was added immediately (16). The GLP-1 bioassay was carried out as previously described (21). Briefly, HEK293 cells were grown in 96-well plates in DMEM, containing 10% fetal bovine serum, to a density of about 5000 cells/well (37°C, 5% CO2 humidified atmosphere). Transfection of these cells was performed in 50 μl of serum-free DMEM per well, containing 0.35 μl Lipofectamine reagent, following the manufacturer’s instructions (Invitrogen). Three cDNAs (5 ng/well each), encoding either the GLP-1 receptor or vector control (pcDNA1) in addition to a multimerized cAMP-responsive promoter linked to luciferase and β-galactosidase, were present in the transfection mix (21). After overnight incubation, 10 μl of mouse serum were added to the wells, and the incubation was continued for an additional 4 h. The medium was then aspirated, and 50 μl of Steady Light Plus reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added. Luciferase activity was determined in a Topcount plate reader. The cell lysates were subsequently further incubated for 1–2 h with o-nitrophenyl-β-D-galactopyranoside [4 mg/ml in phosphate buffer (pH 7.5); Sigma, St. Louis, MO]. Substrate conversion, as a measure...
of β-galactosidase activity, was determined at 420 nm in a Spectra-Max plate reader (Molecular Devices, Sunnyvale, CA).

These data were used to normalize luciferase activity in each well. The amount of GLP-1 bioactivity in serum samples was determined by correcting luciferase readings in GLP-1 receptor-expressing cells by corresponding values observed in vector-transfected cells (no receptor expression). The data were converted to concentrations of bioactive GLP-1 equivalents, using a standard curve measured with synthetic GLP-1(7–36) amide (American Peptide Co., Sunnyvale, CA). The approach of using cognate receptor activation as a readout for hormone concentration ensures that, unlike many immunassays, only bioactive forms of peptide were detected (22). Data conversions were done by nonlinear fitting to the standard curve, using the GraphPad Prism program (version 3.0; San Diego, CA).

Glucose and insulin tolerance tests

NGN3-DPP2 kd mice and appropriate age-matched control littermates were fasted for 18 h before the glucose tolerance test (OGTT). Basal fasting glucose level was measured, and then mice were gavaged with glucose (2 g/kg). Blood glucose levels were measured 20, 40, 60, and 120 min after challenge, using Free-Style glucometers and strips (Abbott Diabetes Care Inc., Alameda, CA). For the insulin tolerance test (ITT), mice were fasted 6 h before an ip injection of 1 U/kg insulin, and blood glucose concentration was measured at baseline as well as 15, 30, 60, and 120 min after the challenge, using FreeStyle glucometers and strips.

Plasma insulin assay

Two hundred microliters tail blood were collected in the morning (random baseline) and 15 min after glucose challenge. Plasma was analyzed for insulin levels, using the UltraSensitive mouse insulin ELISA kit (Crystal Chem, Inc., Chicago, IL), per the manufacturer’s instructions.

Food intake measurement

All experimental mice were fed either regular chow diet (10% kcal fat), referred to as low-fat diet (LFD), or high-fat diet (HFD; 60% kcal fat; D12492; Research Diets, New Brunswick, NJ) ad libitum. Animals were placed individually in bedding-free cages and received preweighed quantities of diet. Intake was measured every 10 d at 4 and 7 months of age.

Home cage locomotor activity

Animals were placed individually into standard plastic housing cages with food and water available ad libitum. Lighting was maintained on a reverse 12-h light, 12-h dark cycle. Motor activity was monitored with the SmartFrame cage rack system (Hamilton/Kinder, Poway, CA). This system consists of 24 PC-interfaced horizontal photobeam frames. The frame (containing 12 photocells; 8 long × 4 wide) surrounds one home cage environment and continuously tracts the animal’s movement. Data were collected in the form of photobeam breaks as an indication of activity within different predetermined zones in the home cage, using MotorMonitor software (Hamilton/Kinder).

Metabolic chamber experiments

Energy intake and indirect calorimetry were obtained for mice (n = 4–5/group, 7 months of age; TSE Calorimetric Systems, TSE Systems, Inc., Chesterfield, MO). The TSE system simultaneously and continuously monitors food intake, oxygen consumption, and CO2 production. Data were collected for 72 h during which time mice had free access to food and water. The first 24 h were considered an acclimation period and excluded from analyses. Respiratory exchange rate (RER) and heat production were calculated, based on O2 consumption and CO2 production.

Statistical analysis

Statistical analyses were performed using the program GraphPad Prism 4.0. Data from OGTT and ITT were analyzed, using the area under the curve (AUC) method and AUC with corrections for baseline differences. For all other variables, statistical significance between the two groups (kd and control) was calculated using the Student’s two-tailed, unpaired t test. Throughout the study significance was set at P < 0.05. For the metabolic variables, data are expressed as mean ± SEM. Data were analyzed using SAS (version 9.3.1; SAS Institute, Cary, NC). The general linear model procedure was used in conjunction with protected post hoc tests (Tukey’s honestly significant difference).

Pancreas preparation

The pancreas was harvested from the anesthetized mice by opening and displaying the gut so that the first loop of the duodenum and the spleen were evident. These mice had not been perfused to obtain a more complete footprint of the pancreas. The colon was then pulled away to display the head of the pancreas, located behind the colon. Once the whole pancreas was visible, it was disengaged from the stomach beginning at the splenic end, although the spleen was left attached as a handle. The spleen was then cut of its attachments. A cut was made across the pylorus and another across the lower part of the duodenum where the pancreas ended. By holding the duodenum and spleen to limit contact with the pancreas, the pancreas was lifted out with these attached organs until any remaining attachments, blood vessels, and lymphatics could be snipped. On a wax board, the pancreas was spread out and the spleen, duodenum, and excess fat and lymph nodes were dissected off. The pancreas was then blotted lightly, weighed, and placed in anatomic orientation in a labeled plastic cassette that was then placed in 4% paraformaldehyde overnight. This entire process was completed no more than 5 min after the time blood flow was disrupted.

Insulin immunofluorescence

Control and DPP2 ko mice (n = 3 each) were anesthetized with an overdose of pentobarbital (50 mg/kg) and perfused transcardially with 20 ml PBS (pH 7.4), followed by 50 ml 4% paraformaldehyde in phosphate buffer (pH 7.4). The pancreas was dissected out and postfixed in the same fixative and immersed in 25% sucrose solution in PBS overnight. The tissue was mounted with Tissue Tek and sectioned on a cryostat (CM3050S; Leica) at 8 μm thickness. Sections were collected on Superfrost slides (Fisher) and air dried for 1 h at room temperature. Sections were processed for insulin immunostaining. Using a hydrophobic pen, a boundary was marked around the periphery of the sections. Sections were washed in PBS, pretreated with 0.5% Triton X-100 in PBS for 20 min, followed by incubation in 10% normal horse serum in PBS for 20 min. Sections were incubated with...
rabbit polyclonal insulin antiserum (Incstar, Stillwater, MN), diluted 1:1000 in antibody diluent (1% normal horse serum, 0.2% Kodak Photo Flo, 0.08% sodium azide), for 24 h 4 C in a humid chamber, followed by incubation in Cy3-conjugated donkey antirabbit IgG (1:250; Jackson ImmunoResearch) for 3 h at room temperature. After washing in PBS, the sections were air dried in the dark and coverslipped with Vectashield mounting medium with 4',6'-diamino-2-phenylindole (Vector). The entire section of the pancreas, stained for insulin, was captured as multiple consecutive partly overlapping images at ×100 magnification on an ImageXpress Micro (Molecular Devices) automated epifluorescence microscope, using the Scan Slide application module of the control software MetaXpress 2.0. The consecutive images were then stitched into a single image representing the entire section, and the total areas of the section, as well as the β-islets based on the insulin staining, were determined with the MetaXpress 2.0. The ratio of the β-islet area to the total section area was determined. The β-cell mass was determined by multiplying the pancreas weight to the ratio.

Generation of NGN3-DPP2 kd mice

To examine the role of DPP2 in vivo, we used the shRNA technology to produce chimeric mice containing the shDPP2-loxP construct (Fig. 1A) (D. A. Mele, Oakes, B., Huber, B. T., manuscript in preparation). We verified the shRNA sequence on the Dharmacon web site (http://www.dharmacon.com/design-center/designcenter.aspx) to ensure its specificity against murine DPP2. In addition, the shRNA was tested against human DPP2, and other members of DPP group and specificity for murine DPP2 was confirmed (23). These mice were crossed with NGN3-Cre tg mice (15), and a stable line, NGN3-DPP2 kd, was selected, which demonstrated NGN3-restricted down-regulation of DPP2 (Fig. 1D). To visualize the tissue-specific expression pattern of NGN3, we crossed an NGN3-Cre tg mouse with a GFP-loxP mouse that leads to NGN3-restricted GFP expression. As expected (15), strong GFP expression in the ventromedial nucleus (VMN) of the forebrain of these mice and lesser expression in the arcuate nucleus (ARC) was observed (Fig. 1B). Few fine GFP-immunoreactive fibers were seen in the hypothalamic paraventricular nucleus (Fig. 1B). No GFP immunolabeling was observed in the hypothalamus of NGN3-Cre or GFP-loxP mice, which served as negative control (Fig. 1B). In addition, enter endocrine cells in the small intestine and pancreatic islets had high GFP expression, whereas the liver was negative (Fig. 1C). Consistent with these findings, NGN3-DPP2 kd mice had restricted down-regulation of DPP2 mRNA in the pancreas but not the liver or muscle, as determined by qRT-PCR (Fig. 1D).

Active GLP-1 levels are unaltered in NGN3-DPP2 kd mice

To further test our hypothesis that DPP2 could be involved in the regulation of the glucose metabolism by modifying gut incretins/neuropeptides, such as GLP-1, we performed a bioassay for active GLP-1 (21). Blood GLP-1 concentration was measured in NGN3-DPP2 kd and control (NGN3-Cre and shDPP2-loxP) mice after fasting as well as after challenge with oral glucose. Surprisingly, we did not see any differences in active GLP-1 in NGN3-DPP2 kd mice compared with controls (Fig. 2). This observation suggested that GLP-1 is not a target for enzymatic action of DPP2.

NGN-3 DPP2 kd mice present with fasting hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance

Male NGN3-DPP2 kd mice and littermate controls were placed either on LFD or HFD. Animals were started on HFD at 5 wk of age. Glucose homeostasis was assessed in these mice at 4 and 7 months of age. Despite normal levels of active GLP-1 in NGN3-DPP2 kd mice, their fasting glucose levels were significantly higher than those of control littersmates, regardless of diet. The observed hyperglycemia was accompanied by glucose intolerance, as determined by OGTT (Fig. 3A). The phenotype was more pronounced in male mice, which is consistent with the well-known fact that female mice in general have higher glucose tolerance than male mice (24, 25); thus, only males were tested in the follow-up experiments.

Impaired glucose tolerance can be attributed to decreased insulin secretion and/or diminished insulin sensitivity. To assess whether DPP2 inactivation affects insulin secretion and sensitivity, we measured baseline plasma insulin concentration and after an oral glucose challenge. In addition, we performed an ITT. NGN3-DPP2 kd mice had a higher baseline plasma insulin concentration as well as 15 min after glucose challenge than the control mice (Fig. 3B). Thus, despite presenting with elevated levels of endogenous insulin compared with the control mice, NGN3-DPP2 kd mice were not able to maintain normal glucose levels. They were also less sensitive to exogenous insulin (ITT) (Fig. 3C). Nevertheless, mutant mice demonstrated normal histology of the pancreas (supplemental Fig. 1A) as well as normal β-cell mass, i.e. insulin production, in the islet cells, as judged by the total β-cell mass (supplemental Fig. 1B). These data indicate that insulin excretion and/or metabolism may be altered in NGN3-DPP2 kd compared with control mice, as reviewed elsewhere (26). In summary, we have shown that NGN3-
DPP2kd mice develop spontaneous pathologic changes in glucose metabolism at an early age (4 months old), and this condition persists with age and when challenged with a HFD (Fig. 3).

**FIG. 3.** NGN3-DPP2kd mice present with fasting hyperglycemia, glucose intolerance, hyperinsulinemia, and insulin resistance. A, The fasting glucose level is higher, and the glucose tolerance in OGTT is lower in NGN3-DPP2kd mice. Blood glucose concentration in NGN3-DPP2kd and control littermates was measured at the indicated times after administration of 2 mg/kg of glucose by gavage. Combined results from several experiments are shown for each data point (mean ± SEM). The statistical significance for the difference between NGN3-DPP2kd and control groups was calculated, based on AUC, with corrections for baseline differences. \( P < 0.002 \) in LFD; \( P < 0.04 \) in HFD. White circles, Control group; black circles, NGN3-DPP2kd group in all figures. B, NGN3-DPP2kd mice have higher baseline insulin levels and increased production of insulin in response to glucose (Glu) challenge. Plasma concentration of insulin was determined in the morning hours (baseline) and 15 min after glucose challenge. Bars, mean ± SD of data obtained with four animals in each group. *, \( P = 0.041 \) for baseline level; **, \( P < 0.008 \) in response to challenge in both age groups on LFD; ***, \( P < 0.028 \) for baseline insulin in all age groups on HFD; ***, \( P < 0.041 \) for response to challenge in HFD group age 4 months and up. C, Glucose fall in response to insulin in ITT is reduced in NGN3-DPP2kd mice on LFD and HFD diets. A representative experiment (\( n = 4 \) in each group) from three repeats is shown (mean ± SEM). The statistical significance for the difference between NGN3-DPP2kd and control groups was calculated, based on AUC.

**FIG. 4.** A, Food intake was increased in NGN3-DPP2kd mice compared with control littermates (\( n = 10 \) per group in HFD group, \( n = 6 \) per group in LFD group). *, \( P < 0.038 \). B, Total body weight in NGN3-DPP2kd mice and littermate controls. Body weight was monitored weekly throughout the course of the experiment (\( P = 0.935 \)). C, Epididymal adipose tissue mass was increased in NGN3-DPP2kd mice at 7 months of age on LFD (***, \( P < 0.045 \)) and HFD (*, \( P < 0.034 \)); perirenal adipose tissue mass and adipose tissue mass was increased in NGN3-DPP2kd mice on HFD (***, \( P < 0.043 \)), compared with control littermates. Bars, mean ± SD of data obtained with \( n = 3 \) in each LFD group and \( n = 4 \) animals in each HFD group. D, The RER of NGN3-DPP2kd mice (7 months of age, on LFD, \( n = 4–5 \) per group) was significantly higher than those of the control littermates, as measured in metabolic chambers. Data were analyzed using SAS (version 9.3.1).
Increased adipose tissue mass is associated with hyperglycemia and insulin resistance (1). Obesity, especially accumulation of fat within intraabdominal depots, has been linked to an increased risk of developing insulin resistance (28, 29). Surprisingly, at 4 months of age when the NGN3-DPP2 kd mice on the LFD developed alterations in glucose and insulin homeostasis, there was no increase in adipose mass. However, at 7 months of age, we observed an increase in epididymal fat pad weight in NGN3-DPP2 kd mice fed LFD. At this time point, total adiposity was also greater in NGN3-DPP2 kd mice fed a HFD, and this was due to increased growth of the intraabdominal depots (Fig. 4C).

Our observation that the phenotype of hyperglycemia, hyperinsulinemia, and insulin resistance is present as early as 4 months, whereas increased adipose tissue accumulation occurs later (7 months), suggests that the increase in adipose tissue mass is a consequence of the hyperglycemia, hyperinsulinemia, and insulin resistance. When tested in metabolic chambers, the NGN3-DPP2 kd mice had increased food intake, confirming our previous findings (Fig. 4A). Importantly, the mutant mice showed a significantly increased RER compared with the control littermates (Fig. 4D and supplemental Fig. 2B), although equal locomotor activity was observed in these mice (supplemental Fig. 2A). The augmented RER in the mutant mice thus provides an explanation why the body weight of the mutant and control mice was identical, despite the increased food intake in the former.

**Liver steatosis is exacerbated in NGN3-DPP2 kd mice**

Impaired glucose metabolism is often associated with development of liver steatosis (30). Therefore, we investigated lipid accumulation in the livers of NGN3-DPP2 kd and control mice. A mild steatosis was observed in livers of LFD-fed NGN3-DPP2 kd mice by 7 months (Fig. 5B). HFD caused an increase in both liver weight at 4 and 7 months and liver lipid content at 7 months in NGN3-DPP2 kd mice (Fig. 5, A–C). Thus, in this mouse model, impaired glucose metabolism occurs before the onset of liver steatosis in LFD-fed animals, and this phenomenon is exacerbated in HFD-fed animals.

**Discussion**

It has been widely accepted that DPP4 is the main regulator of the incretins (6–8). However, the phenotype we observed in the NGN3-DPP2 kd mice has contributed considerable complexity to the established facts of the hormonal regulation of homeostasis by dipeptidases. The NGN3 promoter targets Cre expression and therefore DPP2 kd to enteroendocrine cells of the gut, pancreas, and appetite-controlling nuclei of the hypothalamus, thus promoting DPP2 kd at both the peripheral (GI tract and pancreas) and central (central nervous system) levels.

Whereas NGN3-DPP2 kd mice had normal baseline and acute (after glucose challenge) levels of GLP-1, their fasting plasma glucose was elevated, and their ability to clear glucose in response to oral administration of glucose was significantly decreased. In addition, plasma insulin concentrations were increased compared with those of...
control littermates. In our current studies, we were unable to determine whether the elevated levels of insulin and glucose observed in NGN3-DPP2 kd mice were due to defects in liver, skeletal muscle, and/or adipose tissue. Future investigation, using hyperinsulinemic clamp studies, will identify which of these tissues are contributing to the observed defect in insulin/glucose homeostasis. However, the architecture of the pancreatic islets was normal in the mutant mice at 7 months of age (supplemental Fig. 1), suggesting that the pancreas is not contributing to this metabolic phenotype.

The phenotype of the NGN3-DPP2 kd mice was also characterized by no difference in body weight but increased food consumption, hepatic steatosis, and fat pad mass by 7 months of age. Metabolic chamber experiments indicated that the mutant mice have increased energy expenditure but no change in activity levels. The increased metabolic rate provides an explanation for the difference in food consumption and lack of difference in total body weight. Although total body weight was not different, by 7 months of age, the NGN3-DPP2 kd mice on either diet had augmented epididymal fat pads, compared with those of control littermates, and total adipose tissue mass was increased in NGN3-DPP2 kd mice on HFD. In addition, liver involvement and induction of liver steatosis are considered typical characteristics of centrally mediated obesity (1), and we observed augmented liver steatosis by 7 months of age on either diet. We speculate that the anabolic actions of insulin are promoting fat accumulation in both adipose tissue and liver. When energy is in excess, glucose in the liver is converted to fatty acids and then triglycerides, leading to lipid accumulation and development of steatosis (30). Overall, insulin resistance was present in NGN3-DPP2 kd mice by 4 months of age and preceded increases in adipose tissue mass and hepatic steatosis. Collectively, our data suggest that DPP2 primarily regulates food intake and glucose metabolism, and this dysfunction ultimately results in increased adipose tissue mass and liver steatosis.

Significantly, most of the DPP activity detected in rat brain extract with the chromogenic substrate, AL-Pro-AMC, and selective inhibitors is due to DPP2 (31). Therefore, the appetite-controlling nuclei of the hypothalamus are attractive sites for potential DPP2 enzymatic activity. The specific substrate(s) of DPP2 that regulates glucose and lipid metabolism has not been identified thus far. Any potential substrate for DPP2 cleavage has to: 1) have the N-terminal dipeptide cleaving motif, 2) share tissue and cellular localization with DPP2, and 3) ideally be known to exist in full and truncated form. Many peptide hormones in the brain and/or endocrine cells fit this description, including the well-known incretins, such as bombesin (32), cholecystokinin and vasoactive intestinal polypeptide, neuropeptide Y, and peptide YY as well as the newly described neuromediators, relaxin-3 (33) and nesfatin (34, 35). Because we observed highest NGN3-driven expression in the VMN of the hypothalamus, the appetite-controlling neuropeptides that are secreted by the VMN are of special interest, such as brain-derived neurotropic factor (36), substance P (37), dynorphin, and pituitary adenylate cyclase-activating polypeptide (38).

The phenotype observed in NGN3-DPP2 kd mice is in stark contrast to that of DPP4 ko mice, which are not obese and have a higher glucose tolerance than the controls (16, 17). Thus, our findings provide the first evidence, suggesting that the two N-terminal dipeptidases, DPP2 and DPP4, may have distinct substrate specificities and functional roles. On the other hand, known inhibitors of these dipeptidases have only partial specificity for either DPP4 or DPP2. This cross-reactivity raises a concern about the use of DPP4 inhibitors for chronic treatment of type 2 diabetes because concomitant inhibition of DPP2 activity may offset the desired improvement in glucose metabolism. Further investigation of the specific DPP2 substrate(s) is essential for dissecting the mechanism underlying the observed NGN3-DPP2 kd mouse phenotype and may help to prevent possible side effects of long-term DPP4 inhibition.

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References

34. Lebrun B, Barohay B, Moysse E, Jean A 2006 Brain-derived neurotrophic factor (BDNF) and food intake regulation: a minireview. Auton Neurosci 126–127:30–38