Effects of high-fat diet feeding on Znt8-null mice: differences between β-cell and global knockout of Znt8

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Hardy AB, Wijesekara N, Genkin I, Prentice KJ, Bhattacharjee A, Kong D, Chimienti F, Wheeler MB. Effects of high-fat diet feeding on Znt8-null mice: differences between β-cell and global knockout of Znt8. Am J Physiol Endocrinol Metab 302: E1084–E1096, 2012. First published February 14, 2012; doi:10.1152/ajpendo.00448.2011.—Genomewide association studies have linked a polymorphism in the zinc transporter 8 (Znt8) gene to higher risk of developing type 2 diabetes. Znt8 is highly expressed in pancreatic β-cells where it is involved in the regulation of zinc transport into granules. However, Znt8 is also expressed in other tissues including α-cells, where its function is as yet unknown. Previous work demonstrated that mice lacking Znt8 globally were more susceptible to diet-induced obesity (Lemaire et al., Proc Natl Acad Sci USA 106: 14872–14877, 2009; Nicolson et al., Diabetes 58: 2070–2083, 2009). Therefore, the main goal of this study was to examine the physiological impact of β-cell-specific Znt8 deficiency in mice during high-fat high-calorie (HFHC) diet feeding. For these studies, we used β-cell-specific Znt8 knockout (Ins2Cre:Znt8loxP/loxP) and whole body Znt8 knockout (Cre-:Znt8loxP/loxP) mice placed on a HFHC diet for 16 wk. Ins2Cre:Znt8loxP/loxP mice on HFHC diet had similar body weights throughout the study but displayed impaired insulin biosynthesis and secretion and were glucose intolerant compared with littermate control Ins2Cre mice. In contrast, Cre-:Znt8loxP/loxP mice became more obese, hyperglycemic, hyperinsulinemic, insulin resistant, and glucose intolerant compared with littermate control Cre- mice. These data show that β-cell Znt8 alone does not considerably aggravate weight gain and glucose intolerance during metabolic stress imposed by an HFHC diet. However, global loss of Znt8 is involved in exacerbating diet-induced obesity and resulting insulin resistance, and this may be due to the loss of Znt8 activity in a tissue other than the β-cell. Thus, our data suggest that Znt8 contributes to the risk of developing type 2 diabetes through β-cell- and non-β-cell-specific effects.

Zinc and Znt8 transporters have been found in a wide variety of tissues. In pancreatic β-cells, insulin is crystalized with zinc, forming dense core granules (36). Studies have shown high expression of zinc transporter 8 (Znt8) on insulin granules (10, 63) and further suggest that Znt8 is the primary transporter of zinc into these structures (39). Interestingly, studies have correlated a Znt8 deficiency-dependent defect in zinc transport into insulin granules with abnormal crystallization of insulin (34, 39, 63). In humans, genome-wide association studies have demonstrated a link between a nonsynonymous polymorphism in the gene coding for Znt8 (SLC30A8) and type 2 diabetes (48, 49, 52, 54, 65). In addition, autoantibodies to Znt8 have been found in type 1 diabetic patients (61). Together, these studies outline a role for Znt8 in the pathophysiology of both type 1 and type 2 diabetes.

Two mutant mouse models were used to characterize the function of Znt8 in β-cells (34, 39, 45, 63). Whole body Znt8 knockout (Cre-:Znt8loxP/loxP) mice have elevated fasting blood glucose and impaired glucose tolerance, which was correlated with lower insulin secretion in vivo (39). β-Cells from Cre-:Znt8loxP/loxP mice have reduced granular zinc content and fewer dense core insulin granules. However, two other studies have shown that global deletion of Znt8 does not impair glucose tolerance in male transgenic mice (34, 45). Since Znt8 mRNA was shown to be expressed in human adipose tissue (53) and blood lymphocytes (43) as well as at the protein level in thyroid follicles (37), the adrenal cortex, and α- (63) and pancreatic polypeptide islet cells (56), a β-cell-specific knockout mouse model (Ins2Cre:Znt8loxP/loxP) was generated to more precisely define the role of Znt8 in β-cells (63). Specific deletion of Znt8 in β-cells was associated with glucose intolerance, reduced first-phase glucose-stimulated insulin secretion in vitro, and reduced insulin processing enzyme transcripts in islets (63).

It has been shown that obesity is tightly linked to type 2 diabetes, as it can be causative of or exacerbatory to the disease process. During obesity, adipose tissue increases its release of cytokines such as TNF-α or IL-6 (51, 60), nonesterified fatty acids (7, 46), and hormones, which overall contribute to insulin resistance (22). β-Cells compensate for this insulin resistance by increasing insulin secretion, thus creating metabolic stress. Data on the role of Znt8 in conditions of metabolic stress are scarce. Genome-wide association studies conducted in Asian and Caucasian populations detected an association between the risk allele rs13266634 carried by the Znt8 gene and body mass index (9, 64). Our initial studies as reported in Nicolson et al. (39) showed that Cre-:Znt8loxP/loxP mice fed with a high-fat high-calorie (HFHC) diet displayed greater body weight gain, higher fasting insulin level, and blood glucose, as well as glucose intolerance and insulin resistance. Another study also revealed that Cre-:Znt8loxP/loxP mice fed an HFHC diet were at greater risk of being glucose intolerant and diabetic (34). Nevertheless, whether deficiency of Znt8 specifically in β-cells confers a greater risk for developing diabetes during HFHC dietary intake remained unknown.

Therefore, in the present study we aimed to identify the role of Znt8 in β-cells during metabolic stress induced by HFHC feeding. In conjunction with the Cre-:Znt8loxP/loxP mice, we fed...
Ins2Cre:Znt8loxP/loxP mice an HFHC diet and performed in vivo and in vitro characterization of their phenotypes.

MATERIALS AND METHODS

Animal care and generation of Znt8 knockout mice. The Animal Care Committee at the University of Toronto approved all experiments. Animals were handled according to the guidelines of the Canadian Council of Animal Care. The Cre-:Znt8 −/− mouse model was generated by Genoway (France) using the following strategy: a Znt8 loxP/loxP mouse line (hybrid C57BL/6J/129sv backcrossed 6 times onto C57BL/6J mice) was bred to a CMV promoter-driven Cre recombinase mouse line (C57BL/6J) to excise the LoxP flanked

Fig. 1. Acute zinc transporter 8 (Znt8) gene deletion in Pdx1Cre:ERZnt8loxP/loxP mice. A: tamoxifen (125 mg/kg, 3 injections every other day) was used to trigger deletion of Znt8 through the CreER system driven by Pdx1 promoter. Tamoxifen activates CreER, promoting translocation of CreER into the nucleus. Then, Cre induces recombination between LoxP sites flanking exon 1 of SLC30A8, leading to excision of the start codon and inhibiting the expression of Znt8. B: genotyping strategy used to identify Pdx1Cre:ERloxP/loxP mice. C: qPCR analysis of the expression of Znt8 in isolated islets of corn oil (CO) and tamoxifen-injected mice (**P < 0.001, n = 3). D: immunohistochemistry experiments were performed on dispersed islet cells obtained from CO and tamoxifen-injected mice labeled for insulin and Znt8. E: % of cells labeled were calculated and summarized (n = 3). Scale bar, 25 μm. F and G: granule morphological analysis of β-cells in CO- and tamoxifen-injected Pdx1CreERloxP/loxP mice (n = 3). F: representative electron micrographs (scale bar, 500 nm). G: quantification of different types of β-cell granules (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001.
genomic region (exon 1 of Znt8) in all cells of the animals. Then, whole body Znt8KO mice (Cre+/-, Znt8flox/flox) were backcrossed onto C57BL/6J mice three times. Over the course of the backcrossing, offspring showing lack of Cre expression and heterozygous Znt8 (Znt8flox/-) were kept. We interbred these heterozygous mice. Cre- Znt8flox/flox offspring were designated as Cre- controls. From the same litter, Cre- and homozygous Znt8flox/- mice were used as whole body Znt8KO mice and termed Cre-Znt8flox/-. Therefore, Cre- mice and Cre-Znt8flox/- mice were littermates and had the same genetic background (39). Our β-cell-specific Znt8KO mouse model was generated by crossing TgN(Ins2-Cre)25Mgn mice (maintained on an hybrid C57BL/6/129J background) to Znt8flox/flox mice (hybrid C57BL/6J/129sv backcrossed six times onto C57BL/6J mice). Then, Ins2Cre+/+ and Znt8flox/flox were interbred for two generations. Offspring expressing the Cre transgene alone were used as control mice (Ins2Cre). From the same litters, offspring expressing the Cre transgene and Znt8flox/flox were used as β-cell-specific Znt8KO mice and termed Ins2Cre: Znt8flox/flox. Therefore, control Ins2Cre mice and Ins2Cre: Znt8flox/flox mice were littermates and had the same mixed genetic background. To generate our inducible β-cell-specific Znt8KO mouse model, Znt8flox/flox mice were crossed to Pdx1CreER mice, which express a tamoxifen-inducible Cre recombinase driven by the insulin gene transcription factor pancreatic and duodenal homeobox 1 (Pdx1) (16, 18). Heterozygous offspring were interbred to generate homozygous-inducible β-cell-specific Znt8KO mice and termed Pdx1CreER: Znt8flox/flox. Pdx1CreER:Znt8flox/flox mice were used as three generations. Mice were genotyped using tail DNA and standard multiplex PCR using flox and cre primers (Fig. 1B). To induce Znt8 gene ablation in Pdx1CreER:Znt8flox/flox mice (Fig. 1A), at week 5 of age, tamoxifen (125 mg/kg) or vehicle (5% ethanol-corn oil, Sigma) was injected intraperitoneally three times every other day (16). The tamoxifen stock solution was prepared by initially soaking the tamoxifen in alcohol. Then 50 mg/ml stock solution of tamoxifen was prepared in 5% ethanol-corn oil at 60°C in a water bath. The solution was shaken vigorously until complete dissolution and filtered with a 0.2-μm syringe filter. Before each injection, tamoxifen stock solution aliquots were thawed at 60°C. All the mice used in our study were age matched and male.

**High-fat diet feeding.** From 6 to 22 wk of age, mice were fed an HFHC diet (kcal%, protein 20, carbohydrate 35, fat 45; D12451 Research Diets). Weekly body weights were monitored. Food consumption of Pdx1CreER:Znt8flox/flox mice was calculated weekly by weighing total food amount available for each cage. After 16 wk on the diet, all mice were euthanized for in vitro experiments.

**Transmission electron microscopy.** Freshly isolated islets were prepared, and images were acquired as previously described (39). Samples were observed under a Philips CM100 electron microscope operating at 75 kV. Dense core, empty, light core (gray), and atypical (rod-shaped) granules were manually counted and quantified (63).

**Immunostaining and confocal microscopy.** Islets were isolated and dispersed as previously described (19, 20). Dispersed islet cells were immunostained for Znt8 and insulin, and images were acquired on a Zeiss confocal microscope using the LSM510 software package as previously described (63). Pancreas and fat were sectioned and labeled with an anti-rat Znt8 polyclonal antibody (1:1,000 dilution) as previously described (63).

**Quantitative real-time PCR.** Quantitative real-time PCR (qPCR) analysis was performed as previously described (19). Primers were designed using Primer Express version 2.0 software (Applied Biosystems) and are listed in Table 1. Data were normalized to mouse β-actin mRNA.

**Oral glucose tolerance test (OGTT).** Following a 6-h, fast glucose (1.5 g/kg body wt) was given by oral gavage, and blood glucose was measured at 0, 10, 20, 30, 60, and 120 min from tail vein blood with a glucometer. Blood was centrifuged at 8,000 rpm for 10 min at 4°C. Supernatant (plasma) was used to measure insulin. Area under the curve was calculated using GraphPad Prism software.

**Insulin tolerance test (ITT).** Following a 4-h fast, insulin (0.75 IU/kg body wt) was injected intraperitoneally, and plasma glucose was measured at 0, 15, 30, 60, and 120 min from tail vein blood with a glucometer.

**Glucon, insulin, and proinsulin measurements.** Blood was collected from fed or 16-h-fasted mice from their tail vein. Plasma insulin and plasma proinsulin were measured using ELISA kits from ALPCO diagnostics and Mercodia (Sweden), respectively. Islet insulin content and plasma glucagon were measured by radioimmunoassay (RIA) kits from Millipore, Canada. HOMA-IR (homeostasis model assessment of insulin resistance) was calculated using the formula: HOMA-IR (mmol/l × μU/ml) = fasting glucose (mmol/l) × fasting insulin (μU/ml)/22.5.

**Islet morphological analysis.** Pancreatic islet morphology was analyzed as described in previous reports (32). Results were normalized to whole slice area (μm²). Islet number per slice was manually counted and normalized to whole slice area (μm²).

**Zinc content measurement.** Cell zinc content was measured as previously described (39). Dispersed islet cells were seeded on coverslips and loaded with 2 μM Zinquin (Mellitech) for 50 min at 37°C in Kreb’s ringer buffer containing (in mM) 130 NaCl, 5 KCl, 2 CaCl₂,

### Table 1. PCR primer sequences used for mouse genotyping and qPCR

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<th>Reverse (5′→3′)</th>
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Znt, zinc transporter.
$1 \text{MgCl}_2$, $5 \text{NaHCO}_3$, $1 \text{glucose}$, and $10 \text{HEPES, pH} 7.4$. Cells were washed and imaged using an Olympus BX51W1 fluorescent microscope controlled with Image Master3 software (PTI) with a high-speed monochromator (PTI, Lawrenceville, USA). Zinquin emission was monitored using 365-nm excitation, 375-nm beam splitter, and 385-nm long-pass filter.

Insulin and glucagon secretion. Islets were isolated as previously described (19, 20). Fresh isolated mouse islets (20/vial) were preincubated for 60 min in a glucose-free Krebs-Ringer-HEPES buffer [125 mM NaCl, 5.9 mM KCl, 1.28 mM CaCl$_2$, 5.0 mM NaHCO$_3$, 25 mM HEPES, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$ and 0.1% (wt/vol) bovine serum albumin]. The islets were then incubated for 90 min in 0, 11.1, and 20 mM glucose. Supernatant was collected, and the cell pellets were lysed with acid-ethanol [75% ethanol containing 1.5% (vol/vol) HCl]. DNA was dried with a centriVap centrifugal vacuum concentrator connected to a cold trap and a pump (Labconco), redissolved in 50 μl of ultrapure water, and quantified with a BioPhotometer Plus spectrophotometer (Eppendorf, Hamburg, Germany). Insulin was measured in the supernatant using a Linco Research RIA kit and normalized to DNA content. Area under the curve was calculated using GraphPad Prism software.

Human islets. Human islets were generously provided by Dr. Tatsuya Kin (Clinical Islet Laboratory, University of Alberta, Edmonton, AB, Canada) under the Transplant Program (26).

In vitro free fatty acid exposure. Oleate and palmitate were complexed to fatty acid-free bovine serum albumin in Krebs-Ringer-HEPES buffer following protocols used in previous studies (28, 42). Then, fatty acid Krebs-Ringer solutions were added to 6 mM glucose-RPMI 1640 culture medium (0.5% wt/vol final fatty acid-free bovine serum albumin). Freshly isolated mouse islets or human islets (150 islets/condition) were incubated for 48 h in oleate (0.4 mM) or palmitate (0.4 mM) or control medium. Control culture medium contained all the ingredients without fatty acids.

**Statistical analysis.** Data are expressed as means ± SE. Significance was determined using Student’s t-test or two-way repeated-measures ANOVA with Tukey-Kramer or Bonferroni’s post hoc test. $P < 0.05$ was considered statistically significant.

**RESULTS**

Cre-:Znt8$^{-/-}$ but not Ins2Cre:Znt8loxP/loxP mice on HFHC diet were more obese. Cre-:Znt8$^{-/-}$ mice were significantly more obese than their control mice (Cre-), as seen with their body weight curve and area under the body weight curve from 7 to 16 wk on the diet ($P < 0.05$ to $P < 0.001$, $n = 11$; Fig. 2, A and B). In contrast, after 7 wk on the HFHC diet, Ins2Cre:Znt8loxP/loxP mice gained less weight than their control Ins2Cre mice, as seen from the area under the body weight curve ($P < 0.05$, $n = 15$; Fig. 2, C and D). To demonstrate that the HFHC diet did have an effect on body weight in our Ins2Cre:Znt8loxP/loxP mice, we compared the weight gain of our mice to previous studies examining control mice fed a normal chow diet. A previous study showed that 19-wk-old C57Bl/6J mice fed a Chow diet had gained on average 21% of their original 6-wk-old body weight (27). By comparison, our 19-wk-old Ins2Cre and Ins2Cre:Znt8loxP/loxP mice fed the HFHC diet gained 84 and 73% of their 6-wk-old body weight, respectively. In addition, three other studies using Ins2Cre mice fed a normal chow diet showed body weight gains below what we report here (11, 47, 58). Therefore, HFHC diet feeding resulted in an appreciable increase in body weight gain compared with Chow-fed mice.

Cre-:Znt8$^{-/-}$, but not Ins2Cre:Znt8loxP/loxP, mice on HFHC were insulin resistant. Similar to mice fed a Chow diet (39), Cre-:Znt8$^{-/-}$ mice fed an HFHC diet for 12 wk were significantly more hyperglycemic ($P < 0.05$, $n = 11$; Fig. 3A) and hyperinsulinemic ($P < 0.01$, $n = 11$; Fig. 3B) compared with control Cre- mice. HOMA-IR showed that Cre-:Znt8$^{-/-}$ mice were more insulin resistant than control Cre- mice ($P < 0.01$, $n = 11$; Fig. 3C), which was confirmed by an ITT performed after 14 wk on the diet ($P < 0.05$, $n = 5$; Fig. 4, C and D). Ins2Cre:Znt8loxP/loxP mice were normoglycemic and secreted significantly less insulin than control Ins2Cre mice (Fig. 3, E and F, $n = 13$). HOMA-IR was also significantly lower in Ins2Cre:Znt8loxP/loxP mice than Ins2Cre mice (Fig. 3, E and F, $n = 13$; Fig. 3G). Fasting plasma glucagon was unchanged in both groups of Znt8-deleted mice compared with their respective controls (Fig. 3, D and H, $n = 9$).

At week 15 on the HFHC diet, glucose tolerance was measured using an OGTT. Cre-:Znt8$^{-/-}$ mice showed glucose
intolerance during OGTT compared with their control Cre-
cmice ($P < 0.01, n = 3$; Fig. 5, A, B, D) compared with control Cre-
cmice. In Ins2Cre:Znt8loxP/loxP mice, insulin-positive area was
significantly reduced, islet number was unchanged, and islet size was significantly smaller compared with Ins2Cre
mice ($P < 0.01, n = 3$; Fig. 5, E, F, H). Interestingly, insulin-positive area was approximately two times bigger in Cre-
cmice (Fig. 5A) than in Ins2Cre mice (Fig. 5E) and may reflect differences in their respective genetic backgrounds.
Ins2Cre:Znt8loxP/loxP and Cre-:Znt8$^\sim$ mice did not show any differences in their glucagon-positive area compared with their respective controls (Fig. 5, C and G, $n = 3$).

Ins2Cre:Znt8loxP/loxP and Cre-:Znt8$^\sim$ mice on HFHC diet have lower granule zinc content and abnormal insulin crystalization. Granular zinc content was estimated using Zinquin after 16 wk on the HFHC diet. As previously reported, both Cre-:Znt8$^\sim$ and Ins2Cre:Znt8loxP/loxP pancreatic islet cells had significantly reduced zinc content than their respective control cells (Cre-:Znt8$^\sim$: $P < 0.001$, Fig. 6, A–C).

Fig. 3. Fasting blood glucose (A, E, I), plasma insulin (B, F, J), HOMA-IR
index (C, G, K), and plasma glucagon (D, H, L) in Cre-:Znt8$^\sim$ (A–D, $n =
n = 11$), Ins2Cre:Znt8loxP/loxP (E–H, $n = 11$), and Pdx1CreER:Znt8loxP/loxP
(I–L, $n = 9$) and their respective control mice fed an HFHC diet for 12 wk and
following a 16-h fast. $*P < 0.05$, $**P < 0.01$.

intolerance during OGTT compared with their control Cre-
cmice ($P < 0.05, P < 0.01, n = 5$; Fig. 4, A and B). Ins2Cre:Znt8loxP/loxP mice showed mild glucose intolerance as they displayed a higher blood glucose excursion curve, and the corresponding area under the OGTT curve was increased compared with their control Ins2Cre mice ($P < 0.05, n = 5$; Fig. 4, E and F). Insulin secretion was measured during the
OGTT and showed consistently lower levels of insulin in the
Ins2Cre:Znt8loxP/loxP mice (not significant; Table 2, $n = 5$).

Cre-:Znt8$^\sim$ but not Ins2Cre:Znt8loxP/loxP mice on HFHC diet displayed $\beta$-cell compensation. Cre-:Znt8$^\sim$ mice fed an HFHC diet for 16 wk had a significantly increased insulin-positive area, islet number, and islet size ($P < 0.05,$

Fig. 4. Oral glucose tolerance test in 6-h-fasted Cre-:Znt8$^\sim$ (A, $n = 5$) Ins2Cre:Znt8loxP/loxP (E, $n = 5$), and their respective control mice after 15 wk on an HFHC diet. Area under the glucose curve for the entire test was calculated (B and F). Insulin tolerance tests in 4-h-fasted Cre-:Znt8$^\sim$ mice after 14 wk on HFHC diet (C, $n = 9$). D: area under the glucose curve for the entire test was calculated. $*P < 0.05$, $**P < 0.01$.
Similarly, both groups of knockout mice showed altered granular morphology with reduced dense core granules and increased abnormal (rod-shaped) granules compared with their respective controls (Cre-:Znt8\(^{-/-}\); \(P < 0.001\), Fig. 6, \(F-H\), \(n = 3\)).

Cre-:Znt8\(^{-/-}\) and Ins2Cre:Znt8loxP/loxP mice on HFHC diet have impaired insulin processing. After 16 wk on the HFHC diet, the average fed plasma insulin levels were similar between Cre-:Znt8\(^{-/-}\) and control mice; however, proinsulin levels were significantly higher in Cre-:Znt8\(^{-/-}\) mice and therefore so was the ratio of proinsulin to insulin (\(P < 0.05\), \(P < 0.01\), Fig. 7, \(A-C\), \(n = 5\)). The average fed plasma insulin levels were similar between Ins2Cre:Znt8loxP/loxP and Ins2Cre mice, and again, proinsulin levels were significantly higher in Ins2Cre:Znt8loxP/loxP mice without modulating significantly the ratio of proinsulin to insulin (\(P < 0.05\), Fig. 7, \(D-F\), \(n = 10\)).

Ins2Cre:Znt8loxP/loxP islets have decreased, whereas Cre-:Znt8\(^{-/-}\) islets have increased, insulin secretion. In vitro insulin secretion was assessed after 16 wk on the HFHC diet. In Cre-:Znt8\(^{-/-}\) islets, we observed significantly increased insulin secretion in the presence of 20 mM glucose (\(P < 0.05\), Fig. 8A, \(n = 6\)) but reduced glucagon secretion at 11.1 and 20 mM glucose compared with Cre-islets (\(P < 0.05\), Fig. 8C, \(n = 6\)). Total insulin content from freshly isolated islets was similar between Cre-:Znt8\(^{-/-}\) and Cre-islets (Fig. 8B, \(n = 10\)). We observed a

### Table 2. Plasma insulin levels during OGTTs performed in Ins2Cre and Ins2Cre:Znt8loxP/loxP mice

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<th>30 min</th>
<th>60 min</th>
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<td>Ins2Cre</td>
<td>4.52 ± 0.58</td>
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<td>4.4 ± 1.46</td>
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<tr>
<td>Ins2Cre:Znt8loxP/loxP</td>
<td>3.34 ± 1.28</td>
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<td>3.32 ± 0.87</td>
<td>2.79 ± 0.92</td>
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Values are means ± SE in ng/ml; \(n = 5\). OGTT, oral glucose tolerance test.
significant decrease in insulin secretion from Ins2Cre:Znt8loxP/loxP islets in the presence of 11 mM glucose ($P < 0.05$, Fig. 8D, $n = 6$), with unchanged glucagon secretion or total insulin content from freshly isolated islets compared with control Ins2Cre islets (Fig. 8, E and F, $n = 6–7$).

Tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice maintain similar body weights. Znt8 mRNA expression was evaluated by qPCR, and we showed an 87% decrease in Znt8 expression in pancreatic islets of tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice ($P < 0.001$, Fig. 1C, $n = 3$). Immunohistochemical analysis of primary islet cells from tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice showed a significant decrease in the number of cells labeled for Znt8 and insulin. Some insulin-negative cells remained labeled for Znt8 and are likely α- or pancreatic polypeptide cells ($P < 0.01$, $P < 0.001$, $n = 3$; Fig. 1, D and E). β-Cells of tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice were analyzed under an electron microscope and showed altered granular morphology with reduced dense core granules and increased abnormal (rod-shaped, gray, and empty) granules compared with their respective corn oil-injected Pdx1CreER:Znt8loxP/loxP mice ($P < 0.05$, $P < 0.001$, Fig. 1, F and G, $n = 3$).

Over the course of the HFHC diet study, tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice gained less weight than the corn oil-injected mice, as seen from the area under the body weight curve (Fig. 2, E and F, $n = 9$). Food consumption was measured weekly in these mice, and there was no change in the feeding rate (data not shown). To evaluate a potential nonspecific effect on body weight and food consumption due to the injections or tamoxifen itself, a C57BL/6J control group was injected with corn oil and tamoxifen and fed the same HFHC diet. Body weight and food consumption was measured weekly over a 16-wk period and did not reveal a detectable difference between the corn oil- and tamoxifen-injected groups ($n = 10$, data not shown). Tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice were normoglycemic and had a tendency to secrete less insulin than control mice (Fig. 3, I and J, $n = 7$). HOMA-IR was unchanged (Fig. 3K, $n = 7$), and fasting plasma glucagon was similar between corn oil- and tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice (Fig. 3L, $n = 9$).
**EFFECTS OF HIGH-FAT DIET FEEDING ON Znt8-NULL MICE**

We and others (34, 39, 63) have shown that the loss of Znt8 in mouse β-cells results in lower granular zinc content and abnormal granules. These defects are now considered hallmarks of β-cell Znt8 deficiency and were observed in Cre::Znt8<sup>−/−</sup>, Ins2Cre::Znt8loxP/loxP, and tamoxifen-injected Pdx1CreER::Znt8loxP/loxP mice in the present study. Recent studies have shown that Znt8 polymorphisms are a potential risk factor for type 2 diabetes (48, 50, 52, 54, 55, 57, 65). Some studies in humans have shown no association between adiposity/dietary fat intake and the SLC30A8 risk allele (38, 41, 44, 59), while two recent genome-wide association studies have shown that the SLC30A8 risk allele confers higher risk of developing type 2 diabetes in a lower-BMI population (64) or nonobese subjects (9). We observed in our Ins2Cre::Znt8loxP/loxP mice that the β-cell-specific deletion of Znt8 seemed to have a protective effect against diet-induced obesity compared with controls. HFHC diet-fed Ins2Cre::Znt8loxP/loxP mice showed some glucose intolerance and reduced insulin secretion, consistent with the same mice on a Chow diet (63), without a vast impairment in glucose homeostasis or onset of obesity. The reduced insulin secretion in these mice may have resulted from the small decrease in the β-cell area and/or an impairment in insulin processing and secretion and was likely responsible for the glucose intolerance observed during the OGTT. Reduced insulin secretion combined with lower body weight confirms the improved insulin sensitivity in these mice, which may have developed as compensation for the lower insulin levels. Interestingly, obesity and plasma insulin are tightly linked, as many studies have shown that hyperinsulinemia in both mice and humans aggravates diet-induced body weight gain and insulin resistance (1–4, 35, 40). An insulin secretion defect and/or reduced insulin due to lower β-cell mass in Ins2Cre::Znt8loxP/loxP mice may have prevented the development of hyperinsulinemia during the HFHC diet, and this may have protected against uncontrolled body weight gain and obesity. The origin of the decrease in β-cell area in the Ins2Cre::Znt8loxP/loxP mice is unclear. It is possible that increased insulin sensitivity may have created a reduced demand for insulin, thus decreasing β-cell mass over time. Alternately, previous studies indicate that zinc deficiency may lead to reduced β-cell viability (15, 25). Thus, we could also speculate that the reduced zinc levels in β-cells of Ins2Cre::Znt8loxP/loxP mice may have resulted in more β-cell death, leading to reduced β-cell area independently of insulin sensitivity. In addition, zinc deficiency in β-cells is known to modulate expression of the insulin genes, genes involved in glucose sensing and insulin biosynthesis in 8-wk-old mice (63). It is therefore possible that a similar gene modulation occurred in our Ins2Cre::Znt8loxP/loxP mice fed over the course of the 16-wk HFHC diet period and induced a decrease in β-cell area. Consequently, these data suggest that β-cell Znt8 deficiency does not necessarily exacerbate diet-induced glucose intolerance and may be providing a protective effect against diet-induced obesity.

However, control Ins2Cre mice are reportedly already glucose intolerant with defects in insulin secretion compared with wild-type mice (31). In our study, we cannot confirm that our Ins2Cre mice are glucose intolerant, as we did not compare them with age matched wild-type mice. To address
the possibility of some glucose intolerance and potential developmental complications or compensatory mechanisms in the Ins2Cre:Znt8loxP/loxP mouse model, we created the Pdx1CreER:Znt8loxP/loxP mice. Tamoxifen injection of Pdx1CreER:Znt8loxP/loxP mice induced a significant deletion of the Znt8 gene in islets as shown by qPCR. Furthermore, co-staining with insulin confirmed the effectiveness of tamoxifen to specifically delete Znt8 in β-cells. When fed our HFHC diet, the body weight curve and area under the body weight curve of corn oil-injected control and tamoxifen-injected Pdx1CreER:Znt8loxP/loxP mice were similar to those of Ins2Cre:Znt8loxP/loxP and their controls, thus confirming that constitutive or temporal β-cell-specific deletion of Znt8 does not significantly impact HFHC diet-induced obesity.

Endogenous expression of the Ins2 promoter in the mouse brain has been demonstrated over the course of the embryo development (13, 14) and at the adult stage in the hypothalamus (12, 17, 29, 62). Pdx1 promoter activity was reported in the hypothalamus as well (62). We found Znt8 transcripts at negligible levels in the hypothalamus of Cre− mice. Therefore, it is unlikely that the phenotype observed in Ins2Cre:Znt8loxP/loxP mice originated from deletion of Znt8 in the hypothalamus. Nevertheless, Ins2 promoter activity was reported in many other extrahypothalamic brain areas. The choroid plexus (30), cortex, caudate putamen, ventral pallidum, substantia nigra,pons (17), and mid- and ventral regions of the brain were all shown to have Ins2 promoter activity (62). Importantly, Znt8 expression was reported in the cortex, as seen in the Allen Mouse Brain Atlas (http://www.brain-map.org) (33). Thus, Znt8 deletion in this extrahypothalamic brain region may have played a role in the mild phenotype observed in our β-cell-specific knockout mice. Future studies should be geared to address these concepts.

As stated above, HFHC diet led to a drastic deterioration of insulin sensitivity and development of obesity in global Znt8 knockout mice in contrast to our β-cell-specific deletion models. Some discrepancies between the Ins2Cre:Znt8loxP/loxP and global knockout models may arise simply due to differences in their genetic backgrounds; however, deletion of Znt8 from non-β-cell tissues is most likely responsible. Although our results thus far suggest a lack of appreciable expression of Znt8 in hypothalamus, adrenal glands, fat, and skeletal muscle, it is probable that Znt8 deficiency in some other subset of cells could lead to the insulin resistance or obesity in the global knockout mice. As we only observed changes in glucagon secretion in vitro, likely as a result of elevated insulin secretion, coupled with the unaltered fasting plasma glucagon levels, it suggests that Znt8 deficiency in α-cells may not play a role in defining the insulin resistance phenotype in these mice. Interestingly, pancreatic polypeptide was previously shown to decrease food intake and increase energy expenditure (5). As Znt8 was reportedly found in pancreatic polypeptide cells, this may be an important point of regulation of energy balance that also needs to be further examined (56).

Cre−:Znt8−/− mice were hyperglycemic, hyperinsulinemic, and glucose intolerant. Interestingly, a previous study also showed that HFHC-fed Cre−:Znt8−/− mice had a higher risk of becoming diabetic, whereas all control mice remained nondiabetic (34). Regardless of whether this oversecretion of insulin is a consequence or a prerequisite of the insulin resistance, the resulting hyperinsulinemia may lead to insulin resistance of the hypothalamus, resulting in overfeeding and obesity. Increased fat mass is a trigger in itself to propagate further insulin resistance due to increased release of cytokines and nonsterified fatty acids (7, 46, 51, 60). The β-cells may have attempted to compensate for this insulin resistance by increasing their mass, which subsequently increased islet size and insulin-secretory capacity. On a similar note, enhancement of glucose-stimulated insulin secretion was also apparent in islets isolated from chow-fed Cre−:Znt8−/− mice (39). Thus, the hyperinsulinemia in our Cre−:Znt8−/− mice may have made them more prone to obesity and worsened the phenotype compared with the control mice.

Fig. 8. Glucose-stimulated insulin secretion from Cre−:Znt8−/− (A, n = 6), Ins2Cre:Znt8loxP/loxP (D, n = 6), and their respective control islets measured at 0, 11.1, and 20 mmol/l glucose. Total insulin content of islets was measured at 0, 11.1, and 20 mmol/l glucose. Islets were collected after 16 wk of HFHC diet. *P < 0.05.
Interestingly, we show that an HFHC diet significantly reduces \( \text{H9252} \)-cell Znt8 gene expression in our mice. A similar decrease in Znt8 was found previously in the pancreata of obese \( \text{ob/ob} \) and \( \text{db/db} \) mice (24, 56), as well as following oleate and palmitate treatment in rat insulinoma cells (21). In the present study, we have shown once again that in vitro treatment with oleate and palmitate causes a significant decrease in Znt8 transcript levels in human and mouse islets. However, the reduction in Znt8 expression observed in pancreata from the Akita mouse, which is diabetic due to endoplasmic reticulum stress without an obese phenotype, suggests that factors other than circulating fat that stress the islet can decrease Znt8 expression (56). To this end, Znt8 gene expression was significantly reduced in islets upon in vitro cytokine treatment. However, not all stressors decrease Znt8 expression, as a recent report failed to see any change in Znt8 gene expression upon incubation in high glucose concentrations (6). The decrease in Znt8 expression that we observed in control mice fed an HFHC diet did not translate into any apparent change in \( \text{H9252} \)-cell morphology or zinc content, which may be the result of compensation by other Znt transporters, such as Znt5, which has been shown to be expressed in the Golgi and on \( \beta \)-cell insulin granules (23). However, such compensation was not previously observed in Cre-:Znt8\(^{-/-}\) or Ins2Cre:Znt8loxP/loxP mice (39, 63).

Interestingly, high glucose is a stimulator of zinc influx transporters 6 and 7 (ZIP6 and ZIP7) expression, which could lead to an increase in the cytoplasmic zinc concentration in \( \beta \)-cells (6). Another recent article demonstrated the toxic effect of zinc in cells (8). Therefore, it is conceivable that the phenotype of mice stressed with an HFHC diet could originate as well from the long-term effect glucose has on ZIP6 and ZIP7 in \( \beta \)-cells, which would result in toxic levels of cytoplasmic zinc.

In summary, we show here that, during HFHC diet-induced metabolic stress, global Cre-:Znt8\(^{-/-}\) mice develop severe insulin resistance and obesity, whereas mice with specific deletion of Znt8 in \( \beta \)-cells do not. We suggest that the obesity in Cre-:Znt8\(^{-/-}\) mice is likely dictated by the presence of hyperinsulinemia, not seen in Ins2Cre:Znt8loxP/loxP mice. Consequently, our study suggests that \( \beta \)-cell-specific Znt8 may not confer a higher risk of developing diabetes associated with obesity.

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DISCLOSURES

Dr. Fabrice Chimienti is employed by Mellitech.

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REFERENCES


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