Down-regulation of zinc transporter 8 in the pancreas of db/db mice is rescued by Exendin-4 administration

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Abstract. Recent human genetic studies have revealed that common variants of the zinc transporter 8 (ZnT-8) gene are strongly associated with type 2 diabetes mellitus (T2DM). ZnT-8 has been suggested as a potential candidate in the regulation of insulin secretion in pancreatic β-cells. In this study, we aimed to explore the expression of ZnT-8 in the development of T2DM. The expression of ZnT-8 was investigated in the pancreas and adipose tissue of homozygous db/db mice compared to heterozygous sibling db/+ mice (n=6-8). Furthermore, the effect of Exendin-4 (an analogue of glucagon-like peptide-1) on ZnT-8 expression was examined in the db/db mice. Exendin-4 or vehicle (control) was administered (i.p., 1 nmol/kg) to diabetic 8-week-old db/db mice daily for 14 days (n=8). The results revealed that ZnT-8 protein levels in the pancreas of db/db mice were reduced, accompanied by a decrease in ZnT-8 mRNA. ZnT-8 mRNA and protein levels were also significantly reduced in the epididymal and visceral fat of the db/db mice. Treatment with Exendin-4 up-regulated ZnT-8 gene expression in the pancreas of the db/db mice, but did not affect its expression in adipose tissue. These findings suggest that ZnT-8 synthesis in the pancreas and adipose tissue is down-regulated in db/db mice. Reduced ZnT-8 production in the pancreas may advance defects in insulin secretion in diabetes. These may be reversed, at least partially, by the administration of Exendin-4.

Introduction

It is well known that zinc is required for insulin biosynthesis and the maturation of insulin secretory granules in pancreatic β-cells. Intracellular zinc homeostasis is strictly regulated by zinc binding proteins and zinc transporters. The transport of zinc into insulin secretory vesicles appears to be mainly conducted by zinc transporter 8 (ZnT-8) (1), indicating a possible role of this zinc transporter in the development of diabetes mellitus.

ZnT-8, a new member of the zinc transporter family that is coded by the SLC30A8 gene, is a 369 amino acid protein. ZnT-8 has been identified as a pancreatic β-cell-specific secretory granule zinc transporter (2,3). Recent studies have shown that ZnT-8 is also expressed in human adipose tissue (4), blood lymphocytes (5), the cubical epithelium that lines thyroid follicles, and the adrenal cortex (6).

The strong linkage of single nucleotide polymorphisms in the ZnT-8 gene with type 2 diabetes mellitus (T2DM) has been reported in studies worldwide (7,8). The at-risk allele of ZnT-8 is associated with reduced insulin secretion stimulated by the intravenous glucose tolerance test, impaired conversion of proinsulin to insulin and fasting hyperglycemia (9-11).

Besides being a genetic marker for T2DM, ZnT-8 has also been found to be one of the autoantigens of type 1 diabetes mellitus (T1DM) (12). An association between the risk allele of ZnT-8 and T1DM has also been reported (13), though contradictory results have been found (14,15).

A further study found that the reduced expression of ZnT-8 by shRNA in a pancreatic β-cell line resulted in decreased insulin content and secretion in response to glucose (16). ZnT-8 knockout mice displayed reduced plasma insulin and glucose-stimulated insulin secretion (17,18). This is further supported by the observation that the overexpression of ZnT-8 in INS-1 cells leads to enhanced glucose-induced insulin secretion (1).

Previous studies have strongly indicated that ZnT-8 may be a key player in the mediation of insulin secretion, and may therefore serve as a potential therapeutic drug target for diabetes treatment. In addition to the pancreas, adipose tissue is a major site of ZnT-8 synthesis (4). However, the regulation of ZnT-8 expression in the pancreas and adipose tissue in T2DM remains to be established.

Glucagon-like peptide-1-(7-36)-amide (GLP-1), a hormone secreted by endocrine L cells of the intestinal tract, has unique insulinotropic and growth factor-like signal transduction properties, which make it a new therapeutic agent for the treatment of T2DM (19). Recently, Magnusson *et al* showed that ZnT-8 is a potential target for the pharmacological manipulation of GLP-1 (20). However, whether GLP-1 affects

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ZnT-8 expression *in vivo* remains unclear. Native GLP-1, which is rapidly degraded by dipeptidyl peptidase-IV (21), has a short half-life of a few minutes. Exendin-4, an analogue of GLP-1, is a stable GLP-1 analogue that reduces blood glucose levels as a result of its ability to activate the GLP-1 receptor (22).

The aim of this study was to examine ZnT-8 expression in the pancreas and adipose tissue and the effect of Exendin-4 on ZnT-8 expression in leptin receptor deficient (db/db) mice and their heterozygous sibling (db/+) mice.

Materials and methods

Animals. Eight-week-old male db/db mice (BKS. Cg-m+/+Leprdb) and heterozygous sibling db/+ mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The animals were housed in a temperature-controlled room with a 12-h light/dark cycle, and were allowed free access to food (standard rodent chow) and water. The final body weight of the diabetic and control mice (n=8 per group) was recorded as 41.2±0.7 and 22.6±0.4 g, respectively (mean ± SEM). Mice were sacrificed by cervical dislocation. The study was conducted in accordance with the principles outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (http://grants1.nih.gov/grants/olaw/), and was approved by the local animal ethics committee of the Third Military Medical University.

Drug administration. Experiments were initiated in 8-week-old male db/db mice. Vehicle (control) or Exendin-4 (Sigma, USA) was injected (1 nmol/kg i.p.) (23) daily at 09:00 h (n=8). Injection on days 0 and 7 was delayed until after the completion of an oral glucose tolerance test or an insulin tolerance test, in order to avoid any short-term drug effects. Injection of Exendin-4 was omitted on day 14. The pancreas and adipose tissue (epididymal and visceral) were rapidly dissected and frozen in liquid nitrogen, then stored at -80˚C until analysis.

Real-time PCR for mRNA quantification. Pancreas and adipose tissue samples were homogenized in TRIzol reagent (Roche Molecular Biochemicals, Mannheim, Germany) and total RNA was extracted following the manufacturer's protocol. RNA was quantified by measuring absorbency at 260 and 280 nm, and the ratio was ≥1.8. The integrity of the RNA was confirmed by visual inspection of the two ribosomal RNAs on an ethidium bromide-stained agarose gel. Total RNA (1 μg) was then reverse transcribed into cDNA using a First Stand cDNA Synthesis kit (Toyobo Co. Ltd., Osaka, Japan) with the Thermo Hybrid Px2 thermal cycler (Thermo, Franklin, MA, USA). β-actin was used as an internal control. PCR primers were designed by Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) based on published nucleotide sequences for mouse ZnT-8 (forward: 5'- TTG GTT TTC ATA CGG CTT CC -3'; reverse: 5'- GAT GCA AAG GAC AGA CAG CA -3'; GenBank accession no. NM_172816); mouse β-actin (forward: 5'- CTA CAA TGA GCT GCG TGT GGC -3'; reverse: 5'- GTC CAG ACG CAG GAT GGC ATG -3'; GenBank accession no. EF_156276.1). Each real-time PCR reaction was carried out in a total volume of 20 μl with Quanti Tect SYBR Green PCR Master Mix (MJ Research, Waltham, MA, USA) according to the following conditions: 2 min at 95˚C, 40 cycles at 95˚C for 10 sec, 57˚C (ZnT-8 and β-actin) for 15 sec and 72˚C for 20 sec, using the ABI PRISM 7700 sequence detection system (ABI, Oyster Bay, NY, USA). After amplification, a melting curve analysis was performed by collecting fluorescence data while increasing the temperature from 65 to 99˚C over 135 sec. The cycle threshold (Ct) values were normalized to the expression levels of β-actin.

cDNA constructs and antibody production. The ZnT-8-ORF and its amino-terminal fragment for antibody production were His-tagged through cloning in pET28a (Invitrogen), using murine-specific primers designed on EST sequences. His-ZnT-8 was expressed in *Escherichia coli*, purified on nickel-nitrilotriacetic acid agarose beads (Qiagen, Germany) and injected into rabbits using standard immunization protocols. The antibody was affinity-purified.

Immunofluorescence staining. Pancreas sections from the db/db and db/+ control mice were subjected to immunofluorescence using 12- to 16-μm cryosections. The cryosections were placed in 1:1 acetone:methanol, pre-chilled to -20˚C and incubated for 10 min or longer at -20˚C. The sections were blocked for 1 h with blocking solution (10% normal serum in PBS), incubated for 2 h with primary antibodies and washed with PBS. The slides were then incubated for 40 min with secondary antibodies, washed with PBS and mounted with UltraCruz™ mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All incubations were performed at 37˚C. The primary antibodies used in these experiments were polyclonal anti-ZnT-8 (this study) with a 1:300 dilution, or polyclonal anti-insulin (Abcam, Cambridge, MA, USA) with a 1:300 dilution. Secondary antibodies were goat anti-mouse IgG or goat anti-rabbit IgG (Tianjin Haoyang Biologic Manufacture, Tianjin, China) with a 1:400 dilution. The sections were counterstained with DAPI (40,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to DNA) for visualization of the nuclei. For the negative controls, only secondary antibodies were included in the incubation, without the primary antibodies.

Images for all histological analyses were captured using a Nikon E800 (Nikon Instruments, Tokyo, Japan) microscope and a Nikon DS-U1 digital camera.

Western blotting. Protein samples were prepared from mouse pancreas and adipose tissue. The samples were diluted in sample buffer (250 mmol/l Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for 10 min. A sample aliquot was separated by SDS-PAGE (10%) and then transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked in 5% skim milk in 0.1% Tris-buffered saline/Tween-20 at room temperature for 2 h, and then incubated with anti ZnT-8 antibody at a dilution of 1:500 overnight at 4˚C. Antibody binding was visualized using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence Western blotting detection system (Santa Cruz Biotechnology). Light-emitting bands were

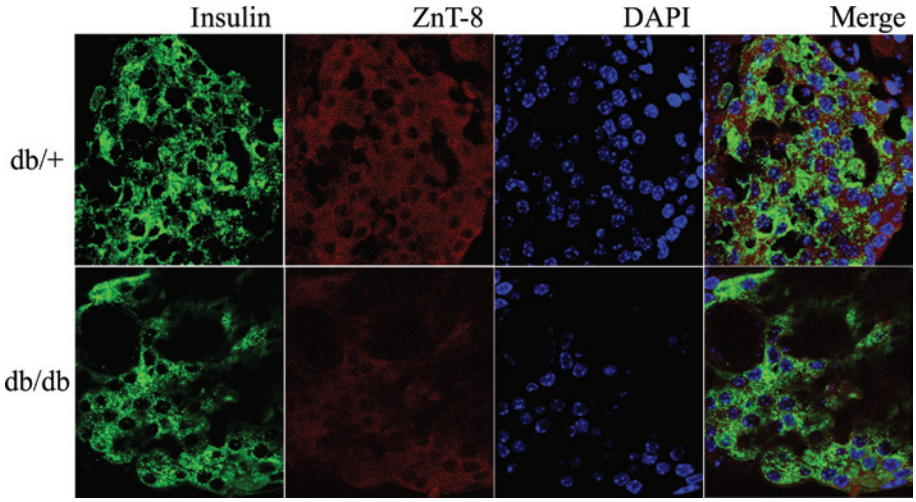

Figure 1. ZnT-8 protein expression in mice pancreatic section. Triple staining was performed for ZnT-8 (red), insulin (green) and DAPI (blue) in mice pancreatic sections from 6 diabetic (db/db) mice and 6 heterozygous sibling (db/+) mice.

detected using X-ray film. The band intensities were quantified using an image scanning densitometer (Furi Technology, Shanghai, China) and were normalized to the β-actin band intensity.

Statistical analysis. Data are expressed as the means ± SEM. Differences between the two groups were analysed by the Student's unpaired t-test. P<0.05 was considered statistically significant.

Results

ZnT-8 protein expression in mice pancreatic section. ZnT-8 protein expression was examined in the pancreatic sections of 6 homozygous (db/db) and 6 heterozygous sibling (db/+) mice. Triple staining for ZnT-8, insulin and DAPI revealed ZnT-8 in the β-cells of the db/+ mice, mainly localized in the cytoplasm (Fig. 1). Notably, ZnT-8 was markedly reduced in all sections from the db/db mice.

ZnT-8 gene and protein expression in the pancreas of db/db mice. To examine whether ZnT-8 expression in the pancreas is altered in T2DM, relative ZnT-8 mRNA levels were determined by real-time PCR in the pancreas of the db/db and db/+ mice. ZnT-8 mRNA levels were significantly reduced (2.5-fold, P<0.01) in the pancreas of the db/db mice compared to the db/+ control mice (Fig. 2A).

ZnT-8 protein expression in the pancreas of the mice was subsequently assessed by Western blotting. ZnT-8 protein levels were consistently reduced by 45% (P<0.01) in the pancreas of the db/db mice compared to the db/+ control mice (Fig. 2B and C).

ZnT-8 gene and protein expression in the adipose tissue of db/db mice. Since the adipose tissue is another major source of ZnT-8 production, ZnT-8 synthesis was further examined in the two major adipose tissue depots (epididymal and visceral) of the db/db and db/+ mice. Both epididymal and visceral ZnT-8 mRNA levels were decreased by 3-fold (P<0.01) and 4-fold (P<0.01), respectively, in the db/db mice compared to the db/+ controls (Fig. 3A and B). In conjunction, ZnT-8 protein levels were also decreased in the epididymal (-28%, P<0.05)

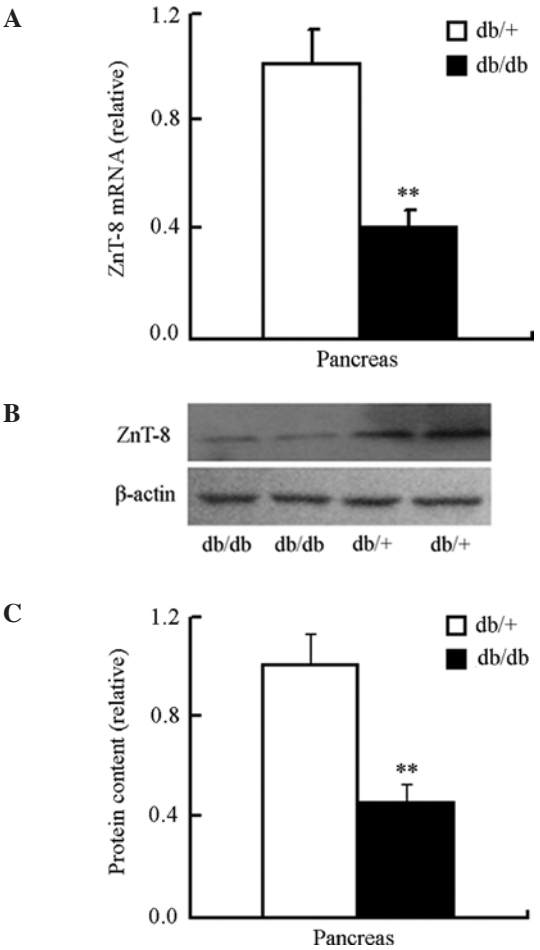

Figure 2. ZnT-8 mRNA and protein expression levels in the pancreas of db/db and db/+ mice. (A) Pancreatic ZnT-8 mRNA levels were measured by real-time PCR and normalized to β-actin. (B) Pancreatic ZnT-8 and β-actin protein levels were detected by Western blotting. (C) Quantification of ZnT-8 was normalised to β-actin. Data are the means ± SEM of each group (n=8). **P<0.01 compared to heterozygous sibling (db/+) mice.

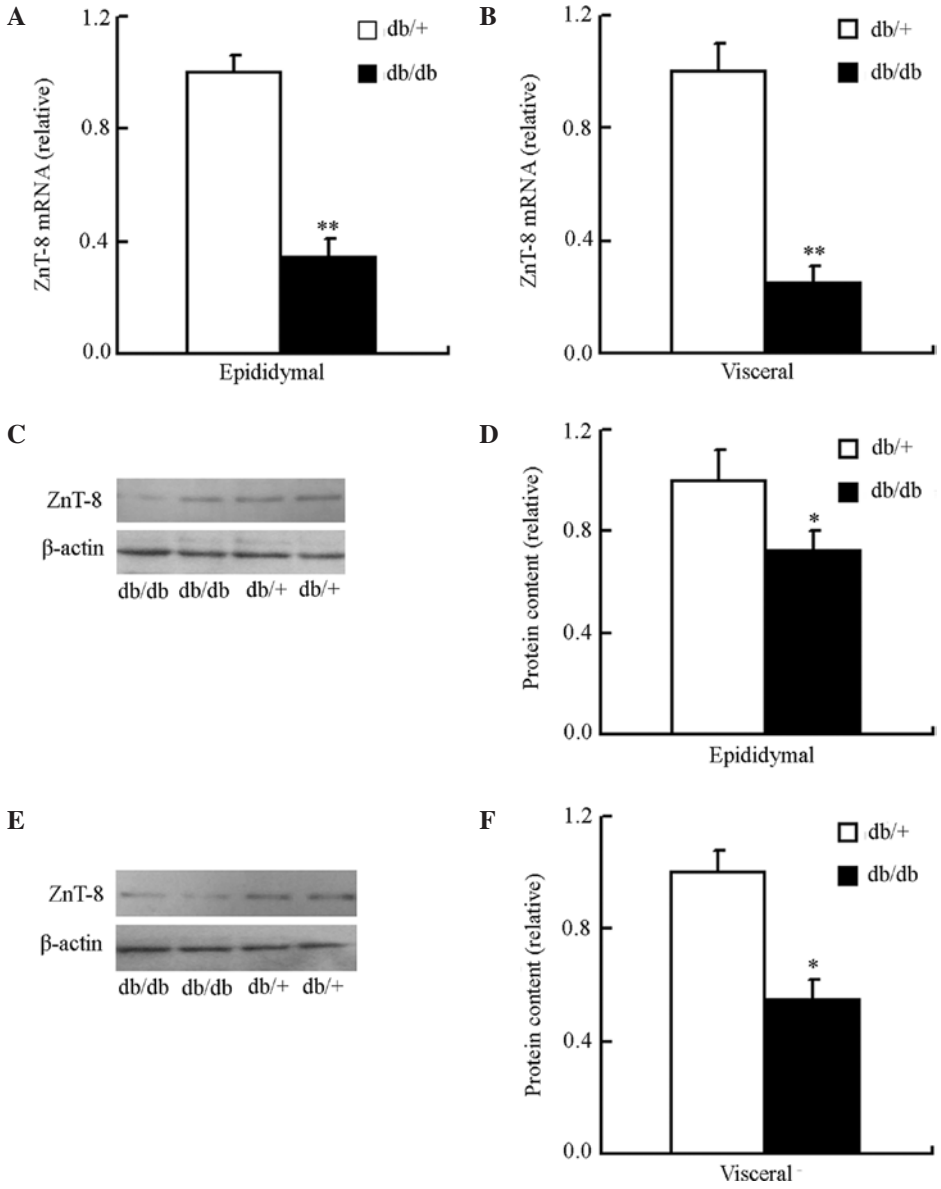

Figure 3. ZnT-8 mRNA and protein expression levels in the adipose tissue of db/db and db/+ mice. Total RNA was extracted from the epididymal and visceral fat of db/db and db/+ mice. (A) Epididymal and (B) visceral fat mRNA levels were measured by real-time PCR and normalized to β-actin. Protein was extracted from the epididymal and visceral fat of db/db and db/+ mice, and ZnT-8 protein amount was detected by Western blotting. Quantification of protein expression was normalized to β-actin in (C and D) epididymal and (E and F) visceral fat. Data are the means ± SEM of each group (n=8). *P<0.05, **P<0.01 compared to heterozygous sibling (db/+) mice.

(Fig. 3C and D) and the visceral (-42%, P<0.05) (Fig. 3E and F) fat of the db/db mice compared to the db/+ mice.

Effects of Exendin-4 on ZnT-8 expression in the pancreas and adipose tissue of db/db mice. The effect of Exendin-4 on ZnT-8 mRNA and protein expression was further examined in the db/db mice. Exendin-4 treatment (1 nmol/kg, i.p.) for 14 consecutive days led to the up-regulation of the mRNA levels of ZnT-8 (2.3-fold, P<0.05) (Fig. 4A). The protein expression of ZnT-8 was also increased (P<0.05) (Fig. 4B and C) in the pancreas of the Exendin-4-treated db/db mice. By contrast, Exendin-4 treatment had no effect on the mRNA levels of ZnT-8 in the epididymal and visceral fat of the db/db mice (both P>0.05) (Fig. 5A and B). The protein expression of ZnT-8 in the epididymal and visceral fat of Exendin-4-treated db/db mice was also unchanged (both P>0.05) (Fig. 5C and D).

Discussion

In T2DM, impaired insulin secretion and decreased β-cell mass can be the end stage outcome of a complex interplay between environmental factors and genetic predisposition. The underlying mechanisms of β-cell failure remain largely unknown. ZnT-8 has recently become the focus of tremendous attention, following the identification of an association between a non-synonymous polymorphism of ZnT-8 and an increased risk of T2DM in genome-wide array studies (7). Thus, ZnT-8 expression and its regulatory mechanisms in T2DM are of great interest to investigators.

Our data show for the first time a significant difference in ZnT-8 gene and protein expression in the pancreas between leptin receptor deficient homozygous db/db mice and heterozygous db/+ control mice. We suggest that the reduced

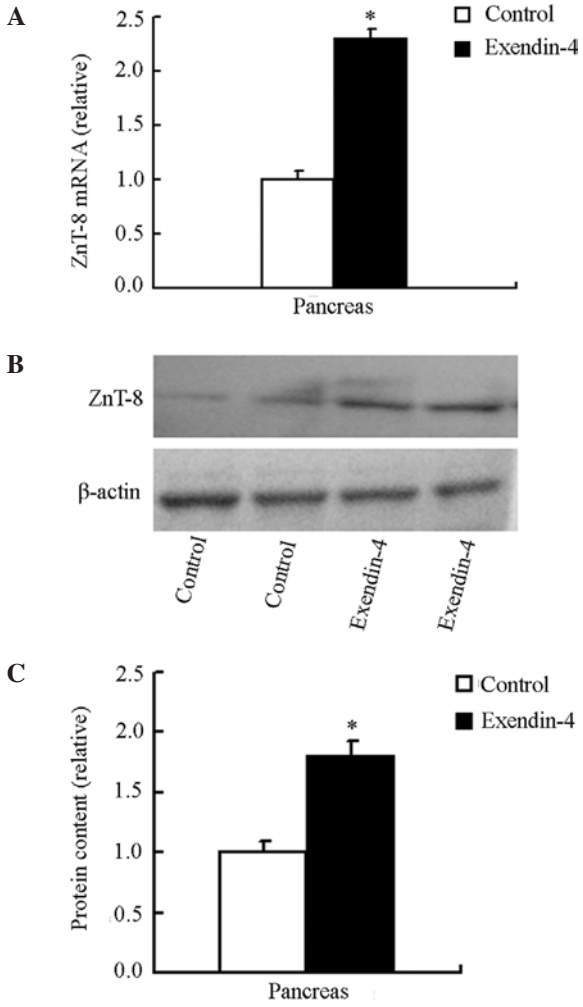

Figure 4. Effects of Exendin-4 on ZnT-8 mRNA and protein expression levels in the pancreas of db/db mice. Vehicle (control) or Exendin-4 was injected (1 nmol/kg, i.p.) daily into 8-week-old male db/db mice for 14 days. Total RNA and protein were extracted from the pancreas of the treated mice. (A) Pancreatic mRNA levels were measured by real-time PCR and normalized to β-actin. (B) ZnT-8 protein amount was detected by Western blotting. (C) Quantification of ZnT-8 was normalized to β-actin. Data are the means ± SEM of each group (n=8). *P<0.05 compared to the control.

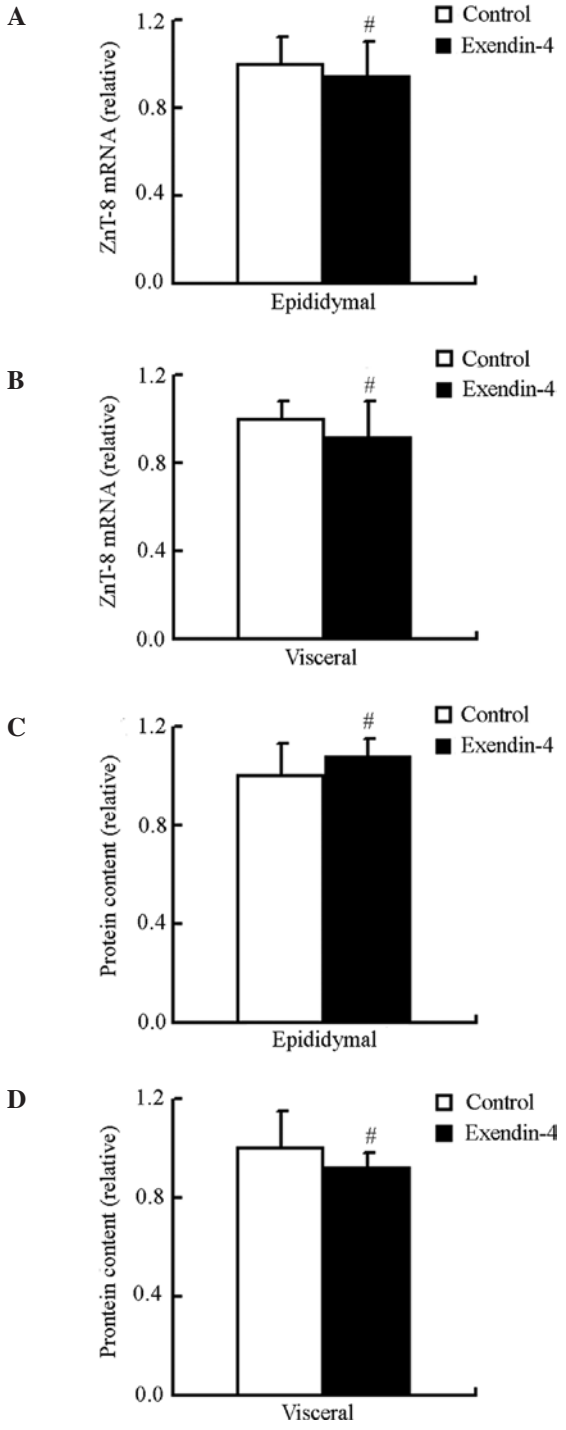

Figure 5. Effects of Exendin-4 on ZnT-8 mRNA and protein expression levels in the adipose tissue of db/db mice. Total RNA and protein were extracted from the adipose tissue of Exendin-4- or vehicle (PBS)-treated db/db and db/+ mice. (A) Epididymal and (B) visceral fat mRNA levels were measured by real-time PCR and normalized to β-actin. Quantification of protein expression was normalised to β-actin in (C) epididymal and (D) visceral fat. Data are the means ± SEM of each group (n=8). P>0.05 compared to the control.

ZnT-8 expression observed in the pancreas of the db/db mice may contribute to the impairment of both insulin biosynthesis and secretion. The mechanism of the contribution of ZnT-8 down-regulation to diabetes requires further investigation.

Emerging studies implicate that ZnT-8 may play a role in glucose intolerance, as ZnT-8 knockout mice are glucose-intolerant, with abnormalities in zinc accumulation, insulin granule morphology and insulin secretion in β-cells, although variations exist between reports (17,24,25). ZnT-8 down-regulation by shRNA in a pancreatic β-cell line mitigated insulin content and secretion in response to glucose, in which cells with fewer dense core granules and less zinc were observed (16). A recent study showed that the overexpression of ZnT-8 inhibits, while knockdown stimulates, glucagon secretion in cultured mouse α-cells (26). Notably, on a high-fat diet, ZnT-8 knockout mice were severely insulin-resistant and more obese than the controls (18), suggesting that ZnT-8 may play a role in peripheral tissues. Thus, glucose intolerance may not solely result from the absence of ZnT-8 in β-cells. Although ZnT-8 was originally thought to be pancreas-specific, studies now suggest that it is also present in human adipose tissue (4).

In adipose tissue, zinc appears to play an important role in a number of processes. In particular, leptin expression is increased in the presence of zinc, while leptin expression and secretion decreases in response to zinc depletion in adipocytes (27-29). Apart from participating in the regulation of leptin secretion, zinc

also seems to be intimately involved in the modulation of adipose tissue release of free fatty acids and glucose uptake in adipocytes, where zinc *per se* functions as an insulinomimetic (30).

In the present study, we also noted a decreased level of ZnT-8 expression in the adipose tissue of the db/db mice compared to the db/+ mice. It is of interest that ZnT-8 has been reported to be expressed at relatively higher levels in subcutaneous compared to visceral fat tissue, and was also found to be relatively much higher in lean compared to obese individuals (4). It is possible that reduced ZnT-8 synthesis in adipose tissue leads to a reduced level of zinc, and consequently affects glucose and fat metabolism.

In the present study, we demonstrated that Exendin-4 (an analogue of GLP-1) up-regulates ZnT-8 gene expression in the pancreas of db/db mice. Therefore, GLP-1 may be a positive regulator of ZnT-8 synthesis in the pancreas. Previously, it was reported that GLP-1 stimulates insulin gene expression and insulin biosynthesis via increased expression and activity of β-cell-specific transcription factor pancreatic and duodenal homeobox gene-1 (PDX1) (31). Based on the evidence that ZnT-8 knockout suppresses a number of transcription factors (including PDX1) that are essential for normal β-cell function (24), it is reasonable to assume that decreased ZnT-8 expression in T2DM leads to the down-regulation of PDX1.

In conclusion, the present study showed that ZnT-8 mRNA and protein expression in the pancreas is down-regulated in db/db mice. It also demonstrated that ZnT-8 mRNA and protein levels are reduced in the adipose tissue of db/db diabetes mice compared to db/+ mice. Additionally, Exendin-4 was found to up-regulate ZnT-8 expression in the pancreas of the db/db mice. Our results suggest that reduced ZnT-8 production may decrease insulin synthesis and secretion via the down-regulation of PDX1 in the pancreas in T2DM. This may be reversed, at least partially, by Exendin-4 treatment.

The findings of the present study provide new evidence regarding the potential importance of ZnT-8 in T2DM. However, the role of reduced ZnT-8 production in the fat of T2DM patients remains unclear. Further investigations are urgently required in order to clarify whether ZnT-8 is a necessary component for Exendin-4-induced insulin secretion, and to determine how Exendin-4 stimulation affects ZnT-8 expression.

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