Regulation and functional effects of ZNT8 in human pancreatic islets

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Abstract

Zinc ions are essential for the formation of insulin crystals in pancreatic β cells, thereby contributing to packaging efficiency of stored insulin. Zinc fluxes are regulated through the SLC30A (zinc transporter, ZNT) family. Here, we investigated the effect of metabolic stress associated with the prediabetic state (zinc depletion, glucotoxicity, and lipotoxicity) on ZNT expression and human pancreatic islet function. Both zinc depletion and lipotoxicity (but not glucotoxicity) downregulated *ZNT8* (*SLC30A8*) expression and altered the glucose-stimulated insulin secretion index (GSIS). *ZNT8* overexpression in human islets protected them from the decrease in GSIS induced by tetrakis-(2-pyridylmethyl) ethylenediamine and palmitate but not from cell death. In addition, zinc supplementation decreased palmitate-induced human islet cell death without restoring GSIS. Altogether, we showed that *ZNT8* expression responds to variation in zinc and lipid levels in human β cells, with repercussions on insulin secretion. Prospects for increasing *ZNT8* expression and/or activity may prove beneficial in type 2 diabetes in humans.

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Introduction

A clear and strong relationship between zinc homeostasis and pancreatic function has been established by numerous investigations over the past decade (Jansen *et al.* 2009). Experimental zinc deficiency is responsible for decreased insulin secretion after glucose stimulation (Chen *et al.* 2000). By contrast, zinc supplementation ameliorates or prevents diabetes in several rodent models of type 1 and 2 diabetes (Taylor 2005). Alteration of zinc homeostasis seems to be also associated with type 2 diabetes (T2D) in humans (Simon & Taylor 2001, Taylor 2005). The highest levels of zinc in the organism are found in β cells where it plays an essential role in insulin crystallization (Emdin *et al.* 1980). Co-secreted zinc has also paracrine effects in pancreatic islets. Zinc is thought to be a positive regulator of K-ATP channels, which are essential for insulin secretion in response to glucose (Ishihara *et al.* 2003). Zinc has also suppressive effects on glucagon secretion (Bancila *et al.* 2004).

The intracellular zinc concentration is tightly regulated by zinc importers (ZIP/SLA39), exporters (zinc transporter (ZNT)/SLC30), and binding proteins (metallothioneins, MT) (Liuzzi & Cousins 2004, Eide 2006, Islam & Loots du 2007). Current understanding of their roles is that ZIP proteins allow intracellular zinc uptake. By contrast, ZNTs are involved in zinc transport from the cytoplasm into either extracellular or intracellular membrane-limited compartments (e.g. secretory vesicles). Briefly, ZNT1 (SLC30A1) is ubiquitously expressed. It is the only transporter known to be involved in zinc efflux across the plasma membrane that confers resistance to zinc (Palmiter & Findley 1995, Liuzzi *et al.* 2001, Cousins *et al.* 2003). ZNT5 (SLC30A5) is expressed more abundantly in pancreatic β cells (Kambe *et al.* 2002). ZNT6 (SLC30A6) transports zinc from the cytoplasm into the *trans*-Golgi apparatus as well as into the vesicular compartments (Huang *et al.* 2002). *ZNT8* (*SLC30A8*) is expressed specifically in α and β cells (Chimienti *et al.* 2004, Gyulkhandanyan *et al.* 2008). It has been localized in the secretory granules, suggesting that ZNT8, along with ZNT5, is involved in supplying zinc for insulin storage (Chimienti *et al.* 2006).

The role of zinc homeostasis in T2D has been recently supported further by genome-wide association studies (GWAS). GWAS consistently reported a strong association between T2D and a nonsynonymous polymorphism (single nucleotide polymorphism rs13266634) within the *SLC30A8*

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gene encoding ZNT8 (Scott *et al.* 2007, Sladek *et al.* 2007, Zeggini *et al.* 2007). Several studies have attempted to elucidate the exact role of ZNT8 in insulin signaling. Fu *et al.* showed that the downregulation of ZNT8 in INS-1 cells reduced both insulin content and glucose-stimulated insulin secretion index (GSIS) while overexpression had the opposite effect (Chimienti *et al.* 2006, Fu *et al.* 2009). Surprisingly, global knockout in mice demonstrated that ZNT8 is not mandatory for glucose control (Lemaire *et al.* 2009, Nicolson *et al.* 2009, Pound *et al.* 2009). However, deficient mice displayed age-, gender-, and diet-dependent glucose intolerance (Lemaire *et al.* 2009, Nicolson *et al.* 2009). ZNT8 β-cell-specific knockout mice (*Znt8BKO*) have reduced expression of key β-cell transcription factors *PDX1* and *MAFA* and of insulin-processing enzymes PC1, PC2, and CPE (Wijesekara *et al.* 2010). *Znt8BKO* islets displayed reduced first-phase GSIS and these mice are glucose intolerant. Although not always concordant, all these studies support the view that ZNT8 may influence insulin signaling in humans.

In order to gather information on zinc effects in human pancreatic islets, we first examined the expression patterns of different ZNTs and islet function under metabolic stress associated with a prediabetic state (e.g. zinc depletion, glucotoxicity, and lipotoxicity). We showed that zinc depletion and lipotoxicity downregulated *ZNT8* expression, which correlated with an increase in apoptosis and a decrease in GSIS. *ZNT8*-overexpressing islets were protected from both zinc depletion and lipotoxicity-related impaired GSIS but not from apoptosis. In addition, we showed that zinc supplementation decreased palmitate-induced human islet cell death without restoring GSIS.

Materials and Methods

Human islet processing

Sixteen human pancreases were harvested from adult brain-dead donors in accordance with French Regulations and with the local Institutional Ethical Committee 'Comité d'Ethique du Centre Hospitalier Régional et Universitaire de Lille'. Pancreatic islets were isolated after ductal distension of the pancreas and digestion of the tissue with Liberase as described previously (Vantyghem *et al.* 2009, Kerr-Conte *et al.* 2010). All experiments were carried out on at least three different donors of $>80\%$ purity.

Cell culture and transfection

Purified islets were cultured in CMRL 1066 medium (Gibco BRL, Life Technologies) containing 0·6% free fatty acid BSA (Roche Diagnostics), penicillin (100 μIU/ml), and streptomycin (100 μg/ml). Zinc deprivation was achieved using tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN 20 μM) for 18 h.

Lipotoxicity was induced by a sodium palmitate treatment for 48 h, using palmitate bound to fatty acid-free BSA prepared as described (Roche *et al.* 1999, Vandewalle *et al.* 2008) except that KRBB was replaced by CMRL 1066. The effective palmitate concentration was determined and adjusted to 0·33 mmol/l in the incubation medium (final concentration). The molar ratio of fatty acid to BSA was ∼5. The effective concentration of BSA was also verified in the final dilution of palmitate and found to be about 0·6%. Fatty acid-free BSA was used at the same concentration in the control condition.

Human islet transfections were performed by microporation (Microporator MP100; Digital Bio, Labtech, Paris, France) after accutase (PPA Laboratories GmbH, Linz, Austria) pretreatment (2500 IE incubated at 37 °C for 2 min) as recently described (Lefebvre *et al.* 2010). Transfection experiments were performed between 18 h and 3 days postisolation: the program was two pulses of 1400 V, 20 ms with 1 μg plasmid for 2500 IE for human islets.

Estimation of islet cell viability and metabolism

Islet viability was assessed after dithizone and trypan blue staining on three aliquots of 80 IE per condition.

ATP content was measured in three aliquots of 40 IE per condition by a luminescence ATP detection assay system as described by the manufacturer (ATPlite, PerkinElmer, Courtaboeuf, France).

Insulin content and glucose-stimulated insulin secretion (GSIS)

Insulin content was assayed using an RIA kit (CIS Bio International, Gif-sur Yvette, France) in the lysates of three aliquots of 40 IE per condition. GSIS was determined by static incubations of islets for 1 h with low glucose (2·8 mmol/l, basal) followed by 1 h with high glucose (20 mmol/l, stimulated). Stimulation indexes (GSIS), defined as the ratio of stimulated to basal insulin release, were estimated from five aliquots of 40 IE per replicate.

Determination of DNA fragmentation

The specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of islet lysates (three aliquots of 160 IE per condition) was achieved by quantitative sandwich enzyme immunoassay using mouse monoclonal antibodies against DNA and histones (cell death detection ELISA kit from Roche Molecular Biochemicals).

RNA preparation and real-time PCR

Total RNA was prepared using RNeasyMinikit (Qiagen). Purified RNA was adjusted to 1 μg/μl and its integrity was assessed with the Agilent RNA 6000 chips coupled with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), by visualizing the 18S and 28S ribosomal RNA.

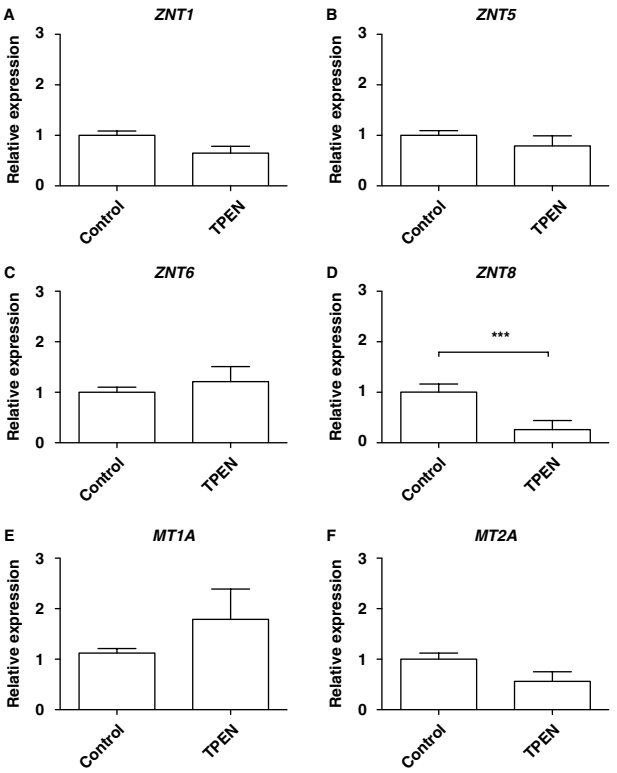

Figure 1 Zinc depletion downregulated *ZNT8* expression in human pancreatic islets. Human pancreatic islets were cultivated in the absence or in the presence of 20 μmol/l TPEN for 18 h. *ZNT, MT1,* and *MT2* expressions were analyzed by real-time PCR and normalized to *RPLO*. Values are mean±S.E.M. of three different pancreases. Significant difference, ***$P<0{\cdot}001$, is indicated.

Reverse transcription (RT) was performed using random hexamers as recommended by the manufacturer (Applied Biosystems). cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosystems) and a mix of RPLO primers and probes. The different probes were purchased from Applied Biosystems (assay on demand kit). Reactions (40 cycles) and data analysis were carried out with an ABI Prism 7700 (PerkinElmer).

Statistical analysis

Results are presented as mean±S.E.M. The statistical differences between the groups are analyzed with ANOVA and the Fisher's least significant difference test using Statview 4.1 software (Abacus, Berkeley, CA, USA).

Results

Zinc chelation downregulated ZNT8 *expression and affected human islet functions*

We first examined the effect of zinc chelation on selected ZNT expressions in human pancreatic islets. For this, islets were isolated by Liberase digestion and density gradient purification and cultured in the absence or in the presence of the zinc-chelating agent TPEN. In preliminary experiments, we found an optimal concentration of 20 μmol/l for 18 h (data not shown). As shown in Fig. 1, human pancreatic islets cultured in the presence of TPEN displayed no significant changes in *ZNT1*, *ZNT5*, *ZNT6* as well as *MT1A* and *MT2A* expression (Fig. 1A, B, C, E and F). We normalized expression levels to housekeeping gene *RPLO*, which was stable under TPEN treatment (data not shown). Interestingly, a 74% decrease in *ZNT8* expression was found. No changes in insulin and *PDX1* expression were observed in these different conditions (data not shown).

We next examined human islet functions in zinc deficiency conditions. We found that TPEN treatment decreased the ATP level (55%) and increased apoptosis (twofold) compared with control islets (Fig. 2A and B). In addition, reducing extracellular zinc concentrations resulted in a significant decrease in GSIS (1·5- vs 1·95-fold) without changing the insulin content (Fig. 2C and D). The effect of TPEN was specific as all metabolic parameters were restored upon zinc supplementation (Fig. 2A, B, C and D). No effect of zinc supplementation alone was observed (data not shown and Fig. 5).

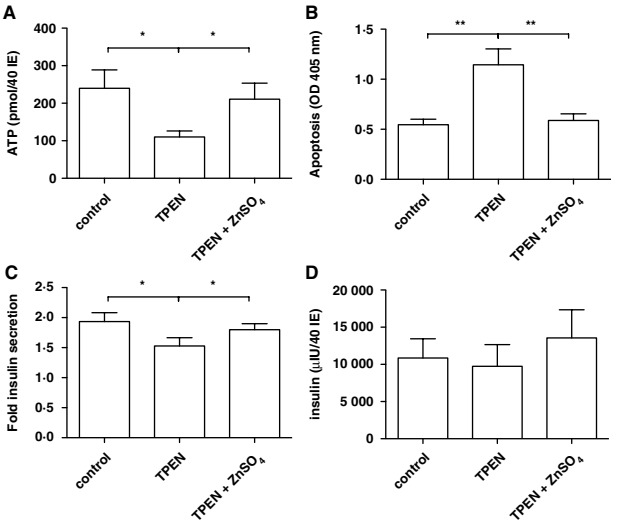

Figure 2 Zinc supplementation restored metabolic parameters of human pancreatic islets treated with TPEN. (A) ATP levels, (B) apoptosis, (C) stimulation index, and (D) intracellular insulin content were measured in human pancreatic islets supplemented or not with 40 μmol/l $ZnSO_4$ and cultured in the absence or in the presence of 20 μmol/l TPEN for 18 h. Stimulation index was calculated as the fold increase in insulin release measured in stimulation over basal levels (insulin release at 20 mmol/l over 2·8 mmol/l glucose for 1 h). Values are mean±S.E.M. of three different pancreases. Significant differences, *$P<0{\cdot}05$ and **$P<0{\cdot}01$, are indicated.

ZNT8 *overexpression restored the stimulation index in human pancreatic islets subjected to zinc chelation*

The data thus far showed a correlation between *ZNT8* expression and altered islet metabolic parameters in the presence of TPEN. To test a possible role of the transporter, we next transfected isolated islets by microporation with plasmids coding for GFP or GFP-ZNT8 and then, 24 h later, subjected them to TPEN treatment. Using this technique, more than 70% of islet cells were transfected and metabolic parameters were not altered (Lefebvre *et al.* 2010). Real-time PCR analysis revealed a 400-fold increase in *ZNT8* expression compared with control islets (data not shown). As shown previously, control islets transfected with GFP plasmid displayed a significant decrease in ATP levels (52%) and GSIS (1·9- vs 2·5-fold) as well as a twofold increase in apoptosis when subjected to TPEN treatment (Fig. 3A, B and C). This effect was not attributable to a change in the insulin storage pool but to an increase in the basal insulin release (Fig. 3C, D, E and F). Overexpressing *ZNT8* did not modify the ATP level and apoptosis observed in the absence or in the presence of TPEN (Fig. 3A and B). *ZNT8* overexpression had no effect on the stimulation index and insulin content in control conditions (Fig. 3C, D, E and F). However, we observed a restoration of GSIS through a decrease in basal insulin release (Fig. 3C, D, E and F).

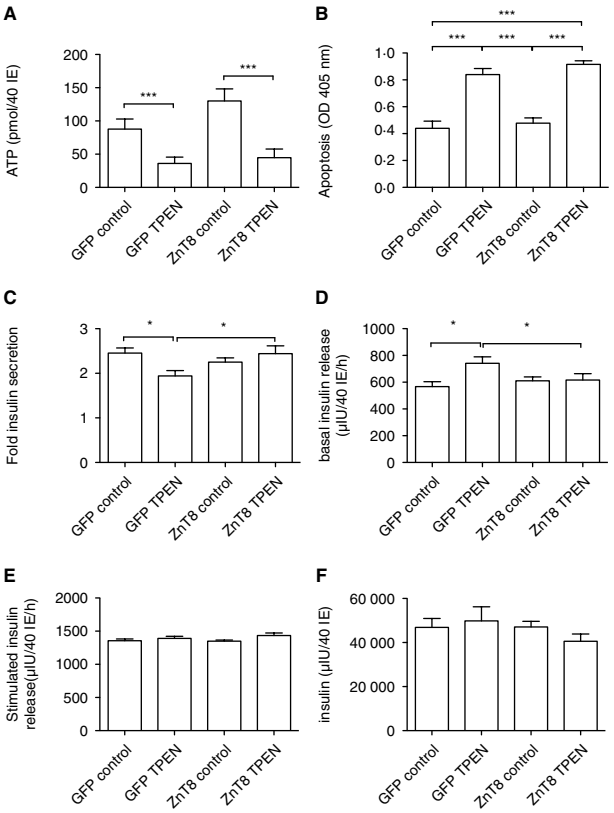

Figure 3 *ZNT8* overexpression restored the stimulation index in human pancreatic islets subjected to zinc depletion. (A) ATP levels, (B) apoptosis, (C) stimulation index, (D) insulin released at 2·8 mmol/l glucose for 1 h (basal insulin release), (E) insulin released at 20 mmol/l glucose for 1 h (stimulated insulin release), and (F) intracellular insulin content were measured in GFP- and *ZNT8*-overexpressing islets cultured in the absence or in the presence of 20 µmol/l TPEN for 18 h. Stimulation index was calculated as described in Fig. 2. Values are mean ± S.E.M. of three different pancreases. Significant differences, $*P<0·05$, $***P<0·001$, are indicated.

Thus, it appears that *ZNT8* overexpression restored the stimulation index by normalizing basal insulin release in the hypozincemic condition but had no effect on TPEN-induced cell death.

Lipotoxicity affected human islet functions independently of extracellular zinc concentrations

Because zinc ameliorates or prevents diabetes in several rodent models, we next investigated the relationship between extracellular zinc concentration and lipotoxicity, a common feature in T2D. We first studied selected ZNT expressions after 48 h of treatment with 0·33 µmol/l palmitate (mimicking lipotoxic condition), supplemented or not with $ZnSO_4$. Real-time PCR analysis revealed that palmitate exposure did not lead to significant changes in *ZNT1* and *ZNT5* expression but significantly increased *ZNT6* (25–30%) expression (Fig. 4A, B and C). We also observed reduced *ZNT8* expression at the mRNA (55%) and protein levels (Fig. 4 D and Supplementary Figure 1, see section on supplementary data given at the end of this article). Zinc supplementation alone or in combination with palmitate increased *ZNT1* expression and tended to increase *MT1* and *MT2* expression (Fig. 4A, E and F). No change in insulin gene expression was seen (data not shown).

We next investigated islet function in these different conditions. As shown in Fig. 5, incubation of islets with palmitate resulted in a significant decrease in the ATP level (60%), insulin content (55%), and GSIS (1·39- vs 2·42-fold; Fig. 5A, C and D). In addition, analysis of DNA fragmentation revealed an increase in apoptosis (Fig. 5B). Supplementation with zinc did not restore the ATP levels, insulin content, or GSIS (Fig. 5A, C and D). However, we found that zinc significantly reduced the apoptosis observed under lipotoxic condition (Fig. 5B).

Taken together, these data show that i) lipotoxicity downregulated *ZNT8* expression and was correlated with altered human islet metabolic parameters and ii) zinc supplementation decreased palmitate-induced human islet cell death.

ZNT8 *overexpression restored the stimulation index in human lipotoxic islets*

We next investigated the effect of *ZNT8* expression on human islet functions under lipotoxic conditions. As shown previously, incubation with palmitate decreased the ATP level (40%) and increased apoptosis (twofold), and *ZNT8*

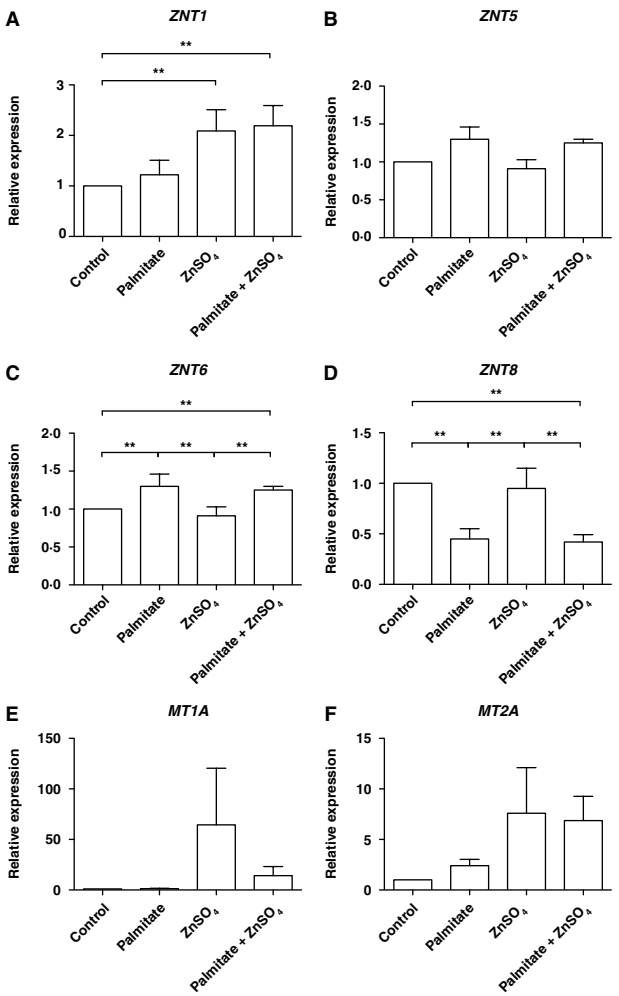

Figure 4 Lipotoxicity downregulated *ZNT8* expression in human pancreatic islets. Human pancreatic islets were cultured in the absence or in the presence of 40 µmol/l $ZnSO_4$ and in the absence or in the presence of 0·33 µmol/l palmitate for 48 h. (A) *ZNT1*, (B) *ZNT5*, (C) *ZNT6*, (D) *ZNT8*, (E) *MT1A* and (F) *MT2A* expressions were analyzed by real-time PCR and normalized to RPLO. Values are mean±S.E.M. of three different pancreases. Significant differences, $^{**}P<0{\cdot}01$, are indicated.

overexpression did not modify the observed results (Fig. 6A and B). As shown previously, palmitate exposure strongly reduced the GSIS (0·95- vs 2·45-fold) through an increase in basal insulin release (2·8-fold; Fig. 6C). Overexpressing *ZNT8* restored the GSIS by normalizing basal insulin release compared with control islets (Fig. 6C, D and E). In addition, *ZNT8*-overexpressing islets displayed a slight but reproducible increase in the GSIS in the presence of palmitate. Interestingly, lipotoxicity decreased the insulin content that was not modified upon *ZNT8* overexpression.

Taken together, these data showed that *ZNT8* overexpression restored the glucose-stimulated insulin secretion under lipotoxic conditions by normalizing the basal insulin release but had no effect on palmitate-induced cell death.

Discussion

In this study, we attempted to elucidate the role of zinc and selected ZNTs in human pancreatic islets in relation to metabolic stress associated with a prediabetic state. Converging evidence suggests that glucose and nonesterified fatty acids contribute to T2D progression through glucotoxic and lipotoxic effects on β cells (Kahn *et al.* 2006). Hypozincemia is also a common feature in T2D and affects β-cell function (Taylor 2005).

We found essentially that ZNT8 is downregulated upon zinc chelation and palmitate exposure. Glucotoxicity and cytokine exposure have been shown to significantly reduce *ZNT8* expression in β-cell lines (Egefjord *et al.* 2009, Smidt *et al.* 2009). However, we did not find any variation in *ZNT8* expression in the presence of different glucose concentrations arguing for a specific regulation in human islets (Supplementary Figure 1). ZNT8 is also downregulated in *db/db* and Akita mice at early stages of diabetes, supporting the relevance of our *in vitro* finding (Tamaki *et al.* 2009).

We next assessed the role of zinc and ZNT8 on human islet survival. We showed that zinc supplementation decreased palmitate-induced islet cell death but did not restore GSIS. However, we did not find any relationship between *ZNT8*

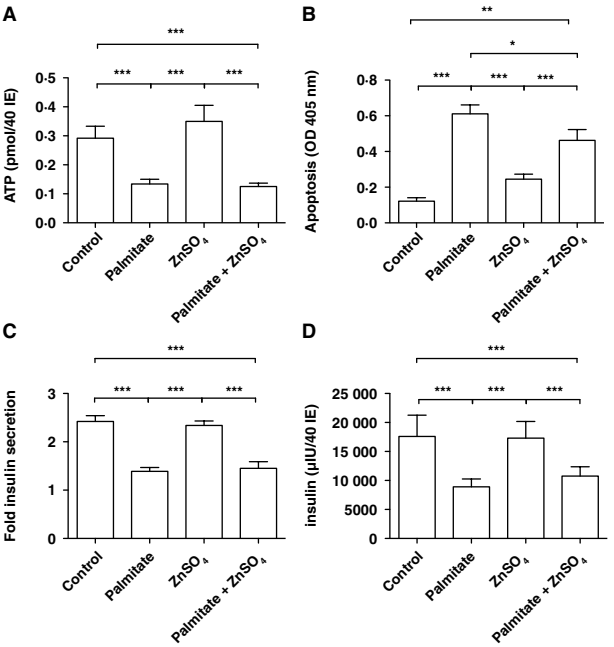

Figure 5 Zinc supplementation protected human lipotoxic islets from apoptosis but did not restore the stimulation index. (A) ATP levels, (B) apoptosis, (C) stimulation index, and (D) intracellular insulin concentration were measured in human pancreatic islets cultured in the absence or in the presence of 40 µmol/l $ZnSO_4$ and in the absence or in the presence of 0·33 µmol/l palmitate for 48 h. Stimulation index was calculated as described in Fig. 2. Values are mean±S.E.M. of three different pancreases. Significant differences, $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$, are indicated.

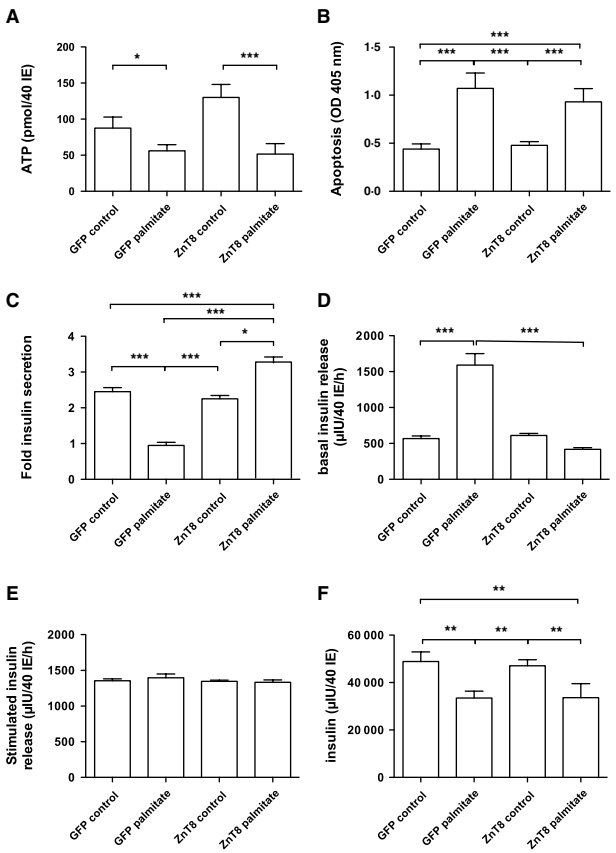

Figure 6 Overexpression of *ZNT8* restored the stimulation index in human pancreatic islets subjected to lipotoxicity. (A) ATP levels, (B) apoptosis, (C) stimulation index, (D) insulin released at 2·8 mmol/l glucose for 1 h (basal insulin release), (E) insulin released at 20 mmol/l glucose for 1 h (stimulated insulin release), and (F) intracellular insulin content were measured in GFP- and *ZNT8*-overexpressing islets cultured in the absence or in the presence of 20 µmol/l TPEN for 18 h. Stimulation index was calculated as described in Fig. 2. Values are mean±S.E.M. of three different pancreases. Significant differences, $*P<0·05$, $**P<0·01$ and $***P<0·001$, are indicated.

expression and apoptosis as *ZNT8*-overexpressing islets were not protected from zinc chelation and lipotoxicity-induced apoptosis. In agreement with these observations, it has been shown that *Znt8* knockdown in primary murine islets did not impact on apoptosis (El Muayed *et al.* 2010). These results are somewhat different from the previous studies performed on β-cell lines. In particular, *Znt8* knockdown in INS-1E cells increased apoptosis, and *Znt8* overexpression protected cells from zinc depletion-induced apoptosis while knockdown increased cell death (Chimienti *et al.* 2006, Petersen *et al.* 2011). The reason for these discrepancies remains unclear but may be related to differences in the physiology of primary islets and INS cells. We recently demonstrated that glutathione peroxidase and superoxide dismutase expressions were decreased by palmitate treatment and further suggested that oxidative stress plays an important role in lipotoxic-induced apoptosis (Vandewalle *et al.* 2008). The observed effect of zinc supplementation could be related to MT induction. These proteins are an important component of the antioxidant protein pool, and they have been shown to have striking effects on the reduction of oxidative stress induced by deleterious conditions found in T2D (Yang & Cherian 1994, Li *et al.* 2004, Yang *et al.* 2008).

We finally demonstrated a direct role of ZNT8 on regulation of basal insulin secretion. ZNT8 downregulation observed under metabolic stress was correlated with an increased basal insulin release while *ZNT8* overexpression reduced it. No changes were observed in insulin content. These results are in agreement with our unpublished data demonstrating that islets isolated from *Znt8*-deficient mice displayed higher basal insulin release then control littermates (Supplementary Figure 2, see section on supplementary data given at the end of this article). Nicolson *et al.* (2009) also observed increased basal insulin release in the same model. This effect is independent of ATP levels that directly affect the exocytotic system. ATP cellular concentration decreased under metabolic stress and remained unaffected by *ZNT8* overexpression (Fujimoto *et al.* 2002, Nabe *et al.* 2006). Further work is needed to elucidate the exact mechanism of how ZNT8 activity impacts insulin release but may be related to a more efficient release of noncrystalline insulin.

In conclusion, our results demonstrated i) a protective effect of zinc supplementation on human islet survival that could involve in part antioxidant activity and ii) a specific role of ZNT8 in insulin release. Its downregulation observed upon metabolic stress associated with a prediabetic state suggests that it might further contribute to T2D progression. Thus, modulating *ZNT8* expression and/or activity may represent a potential therapeutic way to treat T2D.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-12-0071.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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