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ORIGINAL ARTICLE

A low-risk ZnT-8 allele (W325) for post-transplantation diabetes mellitus is protective against cyclosporin A-induced impairment of insulin secretion

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SLC30A8 encodes the β-cell-specific zinc transporter-8 (ZnT-8) expressed in insulin secretory granules. The single-nucleotide polymorphism rs13266634 of *SLC30A8* is associated with susceptibility to post-transplantation diabetes mellitus (PTDM). We tested the hypothesis that the polymorphic residue at position 325 of ZnT-8 determines the susceptibility to cyclosporin A (CsA) suppression of insulin secretion. INS (insulinoma)-1E cells expressing the W325 variant showed enhanced glucose-stimulated insulin secretion (GSIS) and were less sensitive to CsA suppression of GSIS. A reduced number of insulin granule fusion events accompanied the decrease in insulin secretion in CsA-treated cells expressing ZnT-8 R325; however, ZnT-8 W325-expressing cells exhibited resistance to the dampening of insulin granule fusion by CsA, and transported zinc ions into secretory vesicles more efficiently. Both tacrolimus and rapamycin caused similar suppression of GSIS in cells expressing ZnT-8 R325. However, cells expressing ZnT-8 W325 were resistant to tacrolimus, but not to rapamycin. The Down's syndrome candidate region-1 (DSCR1), an endogenous calcineurin inhibitor, overexpression and subsequent calcineurin inhibition significantly reduced GSIS in cells expressing the R325 but not the W325 variant, suggesting that differing susceptibility to CsA may be due to different interactions with calcineurin. These data suggest that the ZnT-8 W325 variant is protective against CsA-induced suppression of insulin secretion. Tolerance of ZnT-8 W325 to calcineurin activity may account for its protective effect in PTDM.
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Introduction

Post-transplantation diabetes mellitus (PTDM) is a serious metabolic complication that occurs after organ transplantation.[1] Patients with PTDM are at a higher risk for long-term cardiovascular events compared with non-PTDM patients.[2,3] In human genome-wide association studies, a non-synonymous polymorphism (rs13266634) in the *SLC30A8* gene was shown to be related to the risk of type II diabetes mellitus.[4–6] Notably, *SLC30A8* encodes the β-cell-specific zinc transporter-8 (ZnT-8).[7] We recently reported that the same allelic variant (which is a

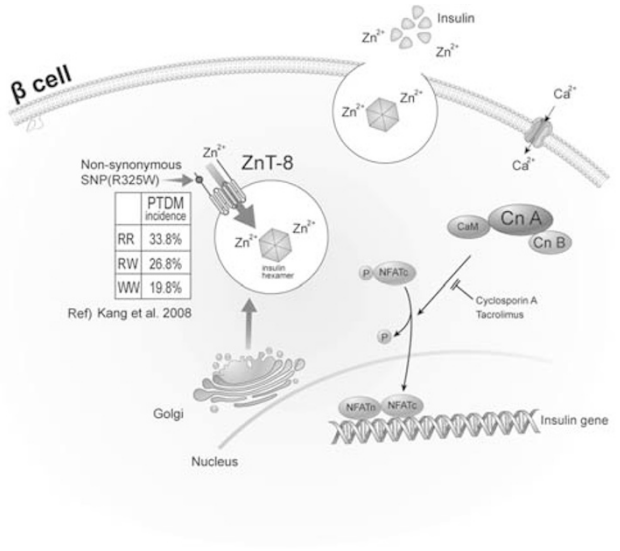

Figure 1 Schematic representation of vesicular ZnT-8 expression and R325W polymorphism in pancreatic islet β-cells. The single-nucleotide polymorphism rs13266634 of *SLC30A8* is associated with PTDM in renal allograft recipients, as demonstrated by Kang *et al.*[8]. *SLC30A8* encodes ZnT-8, which is specifically expressed in β-cells. ZnT-8 promotes zinc accumulation in secretory vesicles. Vesicular zinc is important for the formation of the zinc-insulin hexamer. Calcineurin is the intracellular target of the immunosuppressive drugs CsA and tacrolimus. Calcineurin activity is known to have a role in insulin gene transcription through the regulation of NFATc activity.[16,17]

substitution of tryptophan for arginine at residue 325) of ZnT-8 is also associated with reduced incidence of PTDM in renal allograft recipients[8] (see Figure 1). To date, the molecular basis for PTDM has not been elucidated. Disturbance of insulin secretion has been suggested to be a pathophysiological event that contributes to the development of PTDM.[9,10] The role of ZnT-8 in pancreatic β-cells is also not well characterized. However, ZnT-8 is a plausible candidate factor for modification of glucose metabolism in PTDM because it was shown to colocalize with insulin vesicles in β-cells, and ablation of the gene had a negative effect on insulin secretion in animal experiments.[7,11] On the basis of this evidence, the ZnT-8 polymorphism may be an important variation determining the insulin-secreting capacity of PTDM patients; however, this hypothesis has not been tested at the molecular level.

Zinc has an important role in insulin maturation, storage and secretion.[12] Accumulation of zinc in β-cell secretory vesicles is instrumental in the formation of a zinc-dependent hexameric insulin complex. On glucose stimulation, vesicles fuse with the plasma membrane and subsequently release zinc ions and insulin into circulation. ZnT-8 was identified as a zinc transporter that is mainly present in insulin secretory granules (see Figure 1) to enable cellular efflux of zinc into secretory vesicles.[7,13] ZnT-8 knockout and knockdown experiments *in vivo* and *in vitro* have confirmed that ZnT-8 is important for vesicular distribution of zinc in β-cells.[11,14] However, the precise role of subcellular distribution of zinc by ZnT-8 in pancreatic β-cell physiology is still elusive.

In this study, we tested the hypothesis that ZnT-8 variants produce different effects on insulin secretion in β-cells, in particular, after cyclosporin A (CsA) treatment. CsA is the most widely used immunosuppressive drug in patients undergoing kidney transplantation. However, because of its well-known diabetogenic effect, its utility is sometimes limited. The suggested mechanisms for the development of CsA-induced diabetes are the following: decreased calcineurin/nuclear factor of activated T-cells-dependent insulin gene transcription,[15–17] impairment of priming of the insulin secretory granule,[17,18] decreased β-cell mass[19] and dysfunction of hepatocyte nuclear factor-4α.[20] Although the mechanism by which CsA mediates diabetes is still unclear, CsA ultimately interferes with insulin secretion in β-cells,[21,22] which might contribute to the development of PTDM. Thus, we considered that CsA therapy could be a relevant factor in PTDM, giving rise to different insulin secretion responses to glucose in β-cells depending on ZnT-8 polymorphic variants. We now report the identification of distinct susceptibilities of the ZnT-8 R325W variants to CsA-induced suppression of glucose-stimulated insulin secretion (GSIS) in INS-1E cells.

Materials and methods

INS-1E and MIN6 cell culture and cell counting

INS-1E cells (a rat INS cell line was kindly provided by Dr Claes Wollheim) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. MIN6 cells (a mouse INS cell line) were grown in Dulbecco's modied Eagle's medium supplemented with 15% fetal bovine serum. Both cell lines were cultured in a 5% CO_2 atmosphere at 37 °C. INS-1E cells cultured in 12-well plates were counted using an ADAM automatic cell counter (Digital Bio, Seoul, Korea).

Transfection, drug treatment and insulin ELISA

INS-1E cells were transfected with ZnT-8-R325-EGFP (enhanced green fluorescent protein) (kindly provided by Dr Fabrice Chimienti) or ZnT-8-W325-EGFP (obtained by site-directed mutagenesis from ZnT-8-R325 cDNA) constructs or a mock vector, using the Transfectin reagent (Bio-Rad, Hercules, CA, USA). Cells were then incubated with the KRBH buffer (NaCl 135 mM, KCl 3.6 mM, $NaHCO_3$ 5 mM, NaH_2PO_4 0.5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1.5 mM, HEPES 10 mM and 0.1% bovine serum albumin (pH 7.4)) for 30 min. Cells were stimulated with the KRBH buffer containing glucose (5 or 15 mM) for 4 h. CsA (Sigma, St Louis, MO, USA), tacrolimus (Sigma) or rapamycin (Sigma) was added for 16 h before incubation in RPMI 1640 media, 30 min incubation in the KRBH buffer and 4 h incubation in the KRBH buffer with glucose. For the calcineurin inhibition experiment, a Down syndrome candidate region-1 (DSCR1) C-terminal domain (amino acids 134–197) construct (kindly provided by Dr Young-Jin Cho) was cotransfected with ZnT-8-R325-EGFP or ZnT-8-W325-EGFP. The insulin content of the medium and cell lysates were measured using

a rat/mouse insulin ELISA (enzyme-linked immunosorbent assay) kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Insulin secretion from INS-1E cells after glucose treatment or ZnT-8 variant expression was expressed as that of ZnT-8 R325-transfected INS-1E cells (glucose−/CsA−) set to 100.

Total internal reflection fluorescence microscopy and data acquisition

Images were obtained using a Nikon Eclipse TE200-E inverted microscope (Nikon, Tokyo, Japan) with a ×100 PlanApo total internal reflection fluorescence objective. A 488-nm laser was used for excitation of insulin-EGFP. INS-1E cells expressing insulin-EGFP and ZnT-8 R325 or W325 were cultured in glass-bottom dishes. To monitor the motion of insulin granules, cells were transferred onto a thermostat-controlled stage (37 °C), and then stimulated with 15 mM glucose. Images were acquired every 300 ms. Fusion events were collectively counted from a series of five 1-min sampling sessions at 1, 5, 10, 20 and 25 min after glucose stimulation. Tracking of single granules in different images was performed using the Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

Real-time quantitative PCR

A SYBR Green assay was performed using a standard protocol. cDNA was synthesized by reverse transcription from 2 μg of extracted RNA in a final volume of 100 μl using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR reactions were set up with 2 μl of synthesized cDNA and 10 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl. The primer sequences of rat INS-1 and INS-2 primers used were as follows: forward, AGGTTGCCCGGCAGAAG and reverse, GTTGGTAGAGGGAGCAGATGCT (for INS-1); and forward, GGGAGCGTGGATTCTTCTACAC and reverse, CCACTTGTGGGTCCTCCACTT (for INS-2). The final concentration of each primer was 250 nM. PCR was performed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) using the following cycle conditions: 10 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 40 s at 60 °C and 30 s at 70 °C. The values were normalized to the level of β-actin.

Western blot

The level of ZnT-8 protein expression was analyzed in lysates prepared from INS-1E cells. Samples were electrophoresed using SDS-polyacrylamide gels. After transfer of proteins to a polyvinylidene fluoride membrane, the membrane was blocked with 5% non-fat milk and then incubated with antibody against amino acids 34–49 of human ZnT-8 (Mellitech, Grenoble, France). The membrane was then incubated with a horseradish peroxidase-tagged secondary antibody. After extensive washing in tris-buffered saline with Tween-20, the membrane was developed using the SuperSignal WestPico enhanced chemiluminescent solution (Pierce, Rockford, IL, USA).

^{65}Zn transport assay

Transport of ^{65}Zn into vesicles was measured by the rapid filtration method with modifications.[23] Isolated β-cell vesicles from MIN6 cells were added to the reaction buffer containing 10 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 μCi $^{65}ZnCl_2$ and then incubated at 37 °C for 30 min. The reaction was stopped by filtering the vesicles through a 0.45-μm nitrocellulose filter membrane. After the membrane was washed, the radioactivity of $^{65}ZnCl_2$ was measured using a Wallac 1480 Wizard 3 automatic γ-counter (Perkin-Elmer, Waltham, MA, USA).

Statistical analysis

Data are reported as means ± s.e. Statistical significance was measured by one-way ANOVA (analysis of variance), followed by Bonferroni's post-test.

Results

ZnT-8 variants respond differently to CsA

INS-1E cells transfected with ZnT-8 R325 or ZnT-8 W325 were incubated with increasing concentrations of glucose (5 or 15 mM) to investigate insulin secretion responses with the two variants. Cells expressing either ZnT-8 variant responded to increasing glucose levels with increasing levels of insulin secretion (Figure 2a), indicating that these altered β-cells exhibit glucose-sensitive insulin secretion.[24] Similar to a previous study by Chimienti *et al*.,[13] ZnT-8 overexpression resulted in greater insulin secretion by INS-1E cells than by mock-transfected control cells (Figure 2a), supporting a role for ZnT-8 in the regulation of insulin secretion. As CsA has been shown to lead to deterioration of β-cell function,[21,22] and particularly insulin secretion, we investigated whether ZnT-8 variants show differential susceptibility to CsA with respect to GSIS. CsA-treated INS-1E cells expressing ZnT-8 R325 showed a large decrease in GSIS, whereas cells expressing ZnT-8 W325 showed no such decrease (Figure 2b). The same pattern of responses was reproduced in MIN6 cells and RIN-m5F rat INS cells expressing the ZnT-8 variants (data not shown). CsA itself did not affect basal insulin secretion of either variant (Figure 2c). To examine potential changes in ZnT-8 protein expression levels, the expression of the ZnT-8 variants was measured by western blotting using a ZnT-8-specific primary antibody. No change in the protein expression level of either ZnT-8 variant was observed (Figure 2d). Our results also confirmed that neither the single-nucleotide polymorphism nor CsA treatment affected β-cell growth, which could subsequently affect insulin secretion (Figure 2e).

Next, we investigated the mechanism responsible for the difference in insulin secretion capacity conferred by the polymorphism. We measured the intracellular insulin contents to test whether the polymorphism affects the size of the intracellular insulin pool. The calcineurin pathway is known to regulate insulin gene expression (see Figure 1), and ZnT-8 knockdown reduces the total insulin content in INS-1E cells.[14] We performed an ELISA and found no

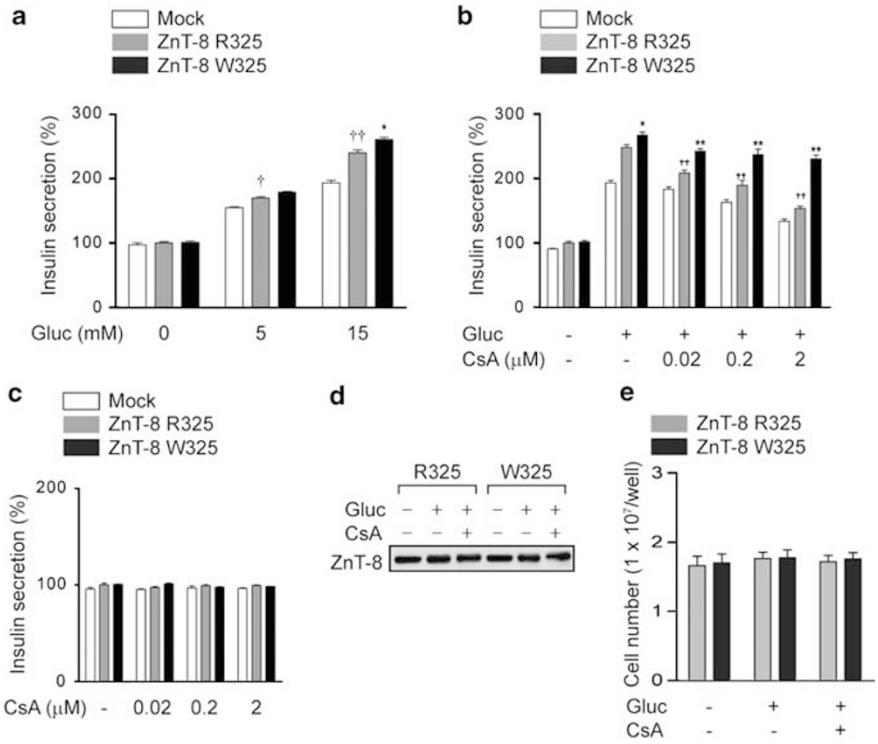

Figure 2 Effect of ZnT-8 R325 and W325 variants on GSIS. INS-1E cells expressing ZnT-8 R325 or W325 variants were used to assess insulin secretion in response to glucose. Control INS-1E cells were transfected with a parental vector that lacks a ZnT-8 variant. After incubation in glucose-free KRBH buffer (see the 'Materials and methods' section), cells were exposed to different concentrations (0, 5 or 15 mM) of glucose for 4 h. The amount of insulin secreted into the medium was measured using ELISA. Statistically significant differences between R325 and W325 were indicated as *($P<0.05$). †($P<0.05$) and ††($P<0.01$) indicate statistical differences between control cells and ZnT-8 R325-transfected cells. (**a**) Cells expressing ZnT-8 R325 or W325 were pretreated with or without 2 μM CsA and then stimulated with 15 mM glucose in the presence or absence of 2 μM CsA. Insulin was measured by ELISA. Statistically significant differences between R325 and W325 were indicated as *($P<0.05$) and **($P<0.01$). $^{++}$($P<0.01$) indicates statistical differences between ZnT-8 R325 (glucose+/CsA−) and ZnT-8 R325 (glucose+/CsA+). (**b**) Basal insulin secretion (without glucose stimulation) from INS-1E cells in the presence or absence of 0.02–2 μM CsA was measured using ELISA. (**c**) Protein expression levels of ZnT-8 variants were analyzed after glucose or CsA treatment by western blot using ZnT-8 antibody. (**d**) Cells expressing ZnT-8 R325 or W325 were counted after glucose or CsA treatment using an automatic cell counter. (**e**) Values of histograms are the mean ± s.e. of at least five experiments. Insulin secretion from ZnT-8 R325-expressing cells (glucose−/CsA−) was set to 100. Data are expressed as the percentage of change in insulin secretion relative to the basal secretion of ZnT-8 R325-expressing INS-1E cells (glucose−/CsA−).

changes in the intracellular insulin content of cells expressing either variant in the presence or absence of CsA (Figure 3a). In addition, measurement of mRNA expression using real-time PCR showed that neither CsA nor high glucose had a measurable effect on insulin mRNA expression, at least in the experimental time frame used for GSIS study (Figure 3b). As the zinc transport activity of ZnT-8 has been positively correlated with insulin-secreting capacity,[11,14,25] we performed a vesicle transport assay to determine whether the polymorphism confers differential zinc transport activity. Vesicles expressing ZnT-8 W325 showed increased transport of ^{65}Zn into vesicles when compared with those expressing ZnT-8 R325 (Figure 4). This is consistent with a previous study showing that ZnT-8 W325 increased the uptake of zinc into secretory vesicles compared with R325, as identified by zinquin fluorescent dye.[25] To further characterize the mechanism by which the ZnT-8 polymorphism affects insulin secretory capacity, we imaged insulin vesicle fusion events using total internal reflection fluorescence microscopy. Secretory vesicles containing EGFP-tagged insulin underwent fusion exocytosis upon glucose stimulation. This process was visually identified in cells as a transition from small fluorescent spots to larger spreading clouds of fluorescence (Figure 5a). Counting these fusion events showed that CsA significantly diminished insulin exocytosis in R325-expressing cells to a greater extent than in W325-expressing cells (Figure 5b).

ZnT-8 variants respond differently to calcineurin

CsA is a well-known inhibitor of calcineurin, and we investigated whether calcineurin inhibition by CsA could account for the suppression of insulin secretion. We compared two immunosuppressive drugs: rapamycin, which suppresses insulin secretion but not calcineurin activity,[26] and tacrolimus, which inhibits both.[16] Cells expressing ZnT-8 R325 that were treated with 5–500 nM tacrolimus (Figure 6a) and 0.02–2 μM rapamycin (Figure 6b) showed a similar pattern of suppression of insulin secretion. However,

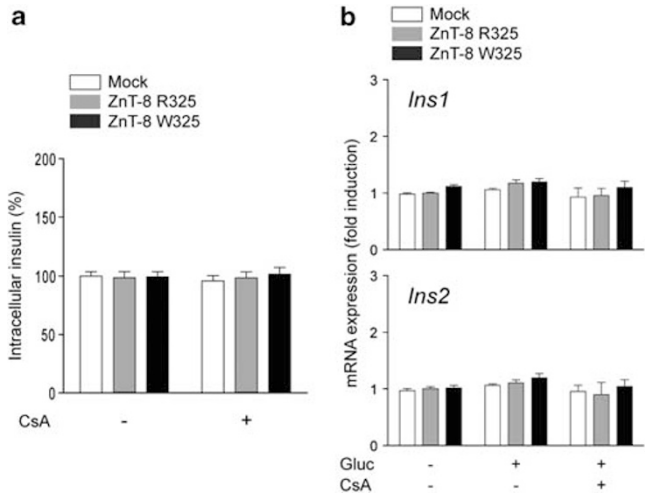

Figure 3 Effects of ZnT-8 R325 and W325 variants on intracellular insulin content and insulin mRNA levels. Intracellular insulin content of INS-1E cells expressing ZnT-8 R325 or W325 variants was measured using cell lysates. Cells were treated with CsA (2 μM) for 16 h. ELISA was performed according to a standard protocol. (**a**) Insulin mRNA expression was measured using real-time quantitative PCR. Cells expressing ZnT-8 R325 or W325 were pretreated with or without 2 μM CsA and then stimulated with 15 mM glucose in the presence or absence of 2 μM CsA. After 4 h of stimulation by glucose, mRNA was extracted from cell lysates and reverse transcribed for subsequent amplification and quantification. Insulin 1 (*Ins1*) and insulin 2 (*Ins2*) mRNA levels were measured by real-time RT-PCR. (**b**) Values of histograms are the mean ± s.e. of at least five experiments.

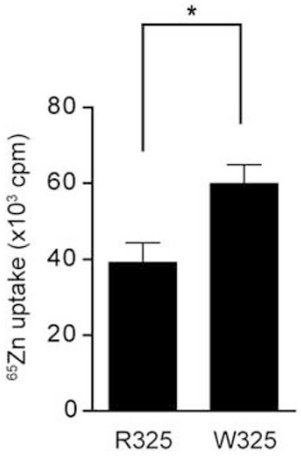

Figure 4 Comparison of zinc uptake ability of the ZnT-8 R325 and W325 variants. Vesicles were isolated from MIN6 cells expressing ZnT-8 R325 or W325. The amount of ^{65}Zn transported into vesicles in 30 min was measured using an automatic γ-counter. Statistical significance is indicated as *($P<0.05$). Values of histograms are the mean ± s.e. of at least five experiments.

cells expressing the W325 variant showed resistance to tacrolimus but not to rapamycin (Figures 6a and b). Concentrations up to 500 nM and 2 μM for tacrolimus and rapamycin, respectively, had no effects on basal insulin

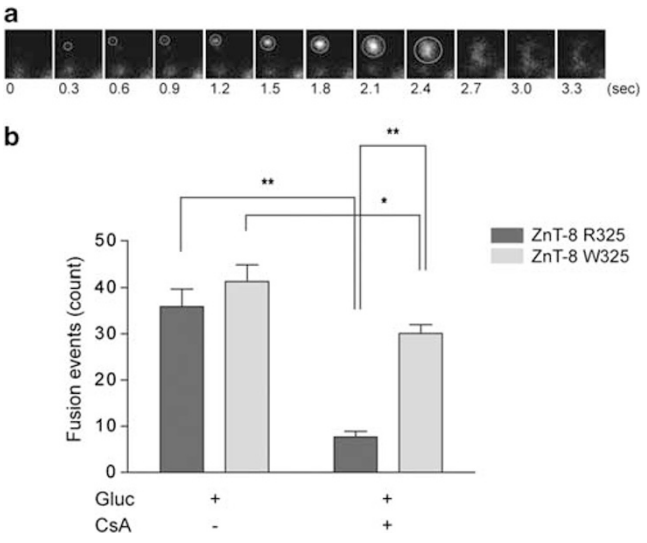

Figure 5 Monitoring of insulin vesicle fusion events using TIRFM. TIRFM was used for tracking of insulin vesicle movement and fusion. A representative insulin vesicle fusion event is demonstrated in serial images obtained at 300-ms intervals. An initial increase in insulin-EGFP fluorescence on vesicle fusion was followed by a subsequent spread of the signal due to granule flattening in the plasma membrane or diffusion of insulin-EGFP upon secretion. (**a**) Cells expressing ZnT-8 R325 or W325 were pretreated with or without 2 μM CsA for 16 h. Fusion events in INS-1E cells expressing ZnT-8 R325 or W325 were manually counted after glucose (15 mM) stimulation in the presence or absence of CsA. Fusion events were collectively counted from a series of five 1-min sampling sessions starting at 1, 5, 10, 20 and 25 min after glucose stimulation and depicted in a bar graph. (**b**) Values of histograms are the mean ± s.e. of at least five independent experiments. Statistical significance is indicated as *($P<0.05$) and **($P<0.01$).

secretion (data not shown). To confirm the role of calcineurin inhibition in CsA-induced suppression of GSIS, INS-1E cells were transfected with an endogenous calcineurin inhibitor, the DSCR1 C-terminal region (amino acids 134–197; DSCR1-CT),[27,28] and GSIS was measured using ELISA. DSCR1-CT inhibited GSIS in cells expressing the R325 variant, but the W325 variant was resistant to the effects of DSCR1-CT overexpression (Figure 6c).

Discussion

The data presented in this study suggest that a single-nucleotide substitution in *SLC30A8* confers resistance to CsA-induced β-cell dysfunction through altering susceptibility to calcineurin inhibition. The unexpected interaction between ZnT-8 and CsA or calcineurin was inferred from our previous human genetic association study.[8] It has long been known that CsA treatment is correlated with the onset of PTDM, for reasons that remain to be elucidated.[3,29,30] The differing effects of two ZnT-8 variants on GSIS suggest that patients undergoing CsA immunosuppressive treatment who have the 'low-risk' ZnT-8 variant (W325) may retain insulin-secreting capacity compared with those who have the 'high-risk' variant; this may account for the protective

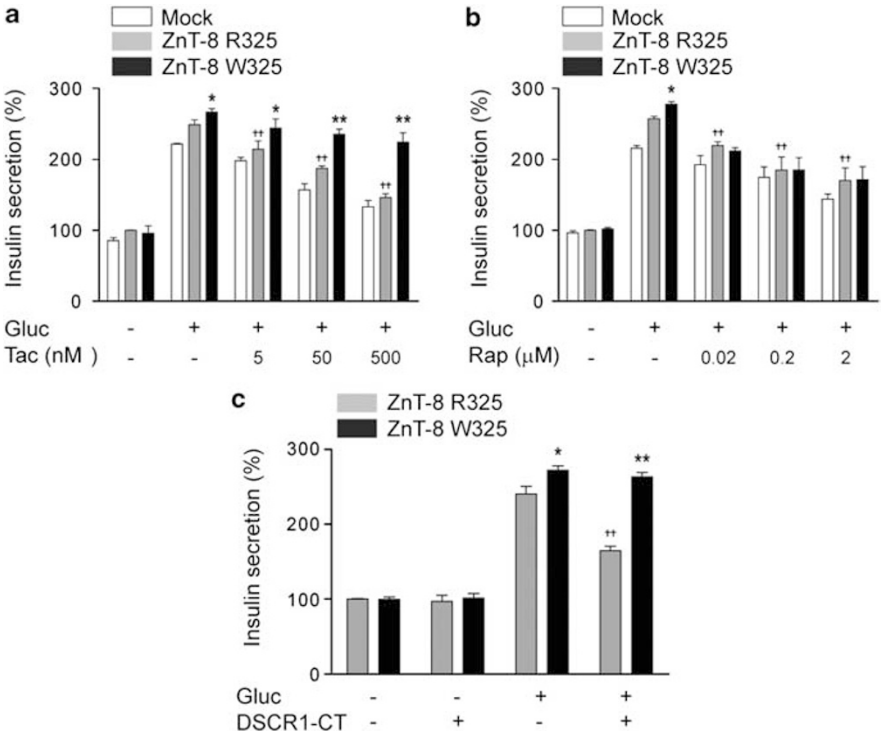

Figure 6 Effects of calcineurin inhibition on insulin secretory responses. Cells were pretreated with tacrolimus (5, 50 or 500 nM) or rapamycin (0.02, 0.2 or 2 μM) for 16 h. Cells expressing either ZnT-8 variant were exposed to glucose for 4 h in the presence or absence of tacrolimus (**a**) or rapamycin (**b**). Insulin secretion into the medium was measured by ELISA. Statistically significant differences between R325 and W325 were indicated as *($P<0.05$) and **($P<0.01$). $^{++}$($P<0.01$) indicates statistical differences between ZnT-8 R325 (glucose + /Tac− or Rap−) and ZnT-8 R325 (glucose + /Tac + or Rap +) (panels a and b). Cells were cotransfected with the DSCR1-CT construct and the ZnT-8 R325 or W325 variant. After 36 h, cells were treated with 15 mM glucose. GSIS was measured using ELISA. Statistically significant differences between R325 and W325 were indicated as *($P<0.05$) and **($P<0.01$). $^{++}$($P<0.01$) indicates statistical differences between ZnT-8 R325 (glucose + /DSCR1 CT−) and ZnT-8 R325 (glucose + /DSCR1 CT +). (**c**) Values of histograms are the mean ± s.e. of at least five independent experiments. The amount of basal insulin secretion of ZnT-8 R325-expressing cells was set to 100. Data are expressed as the percentage of change in insulin secretion relative to the basal secretion of ZnT-8 R325-expressing INS-1E cells.

effect of the W325 allele with respect to PTDM. This also suggests that impairment of insulin secretion by CsA may contribute to PTDM. The differential effects of CsA on GSIS depend on the ZnT-8 R325W polymorphism; this evidence indicates that ZnT-8 is an important regulator of insulin secretion, and that CsA toxicity is closely related to ZnT-8 functions. On the basis of our data, the most likely pathway for CsA in diminishing secretion of insulin involves ZnT-8.

Even in the absence of CsA, a statistically significant effect of W325 variants on GSIS was noted, but the differences were modest. More efficient accumulation of zinc in secretory granules in cells expressing ZnT-8 W325, as demonstrated by previous studies,[13,25] appears to account for this effect. Importantly, the different effects of the two variants on insulin secretion became more pronounced when β-cell insulin secretion was suppressed by CsA. CsA treatment thus provides a unique environment in which we can investigate the effect of ZnT-8 polymorphism on the insulin secretion of β-cells in response to glucose. CsA is the most widely used immunosuppressive drug in kidney transplantation. Thus, the understanding of the molecular mechanisms underlying different susceptibility of cells expressing either ZnT-8 variant to CsA has important clinical utility as well as experimental significance.

It is feasible that either the ZnT-8 polymorphism or CsA might affect ZnT-8 expression levels, resulting in the observed differences in the amount of secreted insulin. Fu *et al*.[14] suggested that altered insulin secretory responsiveness might be due to ZnT-8 polymorphism-associated differences in protein expression levels. The absence of changes in the protein expression of either variant of ZnT-8 in our study indicates that other mechanisms might be involved. CsA might be expected to affect insulin synthesis or insulin degradation, thereby affecting the size of the intracellular insulin pool, because the calcineurin pathway is known to regulate insulin gene expression[16] (see Figure 1), and ZnT-8 knockdown reduces the total insulin content in INS-1E cells.[14] However, this possibility was ruled out by the finding that no measurable changes in insulin protein content or mRNA expression occurred. Rather, the reduction in insulin vesicle fusion events after CsA treatment, as revealed by the total internal reflection fluorescence imaging technique, suggests that CsA may affect certain

steps of insulin granule exocytosis. Taken together, this evidence suggests that β-cells expressing ZnT-8 W325 differ from those expressing the R325 variant in their efficiency in insulin secretion rather than in insulin production; however, the underlying mechanism remains to be elucidated.

One possibility is that ZnT-8 W325 promotes zinc ion transport into vesicles to a greater extent than does ZnT-8 R325. The zinc transport activity of ZnT-8 has been positively correlated with insulin-secreting capacity,[11,14] vesicular zinc is crucial for insulin maturation and secretion.[12] A previous study showed that uptake of zinc into secretory vesicles was increased in cells expressing ZnT-8 W325 compared with those expressing R325, as demonstrated using zinquin fluorescent dye.[25] Although we adopted a different zinc transport assay, the *in vitro* vesicle ^{65}Zn uptake assay, we obtained the same results as those from the zinc fluorescence-based cell assay. This finding strongly supports the conclusion that ZnT-8 W325 is more efficient in promoting zinc ion uptake into insulin vesicles, which might contribute to enhanced insulin secretion. As in previous ZnT-8 knockout or knockdown studies,[11,14] the effects of the zinc transport activity of ZnT-8 on insulin secretion are difficult to assess as a direct cause–effect relationship. Confirming the predicted role of the ZnT-8 variants awaits further studies using mutation of amino acids specifically related to zinc transport activity, which might need further background information about the ZnT-8 structure.

CsA is a well-known inhibitor of calcineurin; the resulting inhibition of phosphatase activity inhibits nuclear factor of activated T-cells-mediated insulin gene transcription. However, the lack of an effect of CsA on insulin mRNA levels in INS-1E cells brings into question whether calcineurin inhibition is related to CsA-induced differential suppression of GSIS in cells that express the two ZnT-8 variants. Tacrolimus, another widely used immunosuppressant drug in organ transplantation, also inhibits calcineurin activity and suppresses insulin secretion.[16] In contrast, rapamycin inhibits the mammalian target of rapamycin without affecting calcineurin activity as well as inhibiting GSIS in β-cells.[26,31] Although the therapeutic ranges of tacrolimus and rapamycin are comparable, the potency of rapamycin in inhibiting insulin secretion seemed to be much lower than that of tacrolimus in this study. This may partly explain why rapamycin has less diabetogenic activity. However, the definite involvement of rapamycin in PTDM is still under investigation.[32,33] Recently, the suppression of carbohydrate metabolism by rapamycin was suggested as a candidate mechanism for the inhibition of insulin secretion.[34] However, the mechanism of action of rapamycin in the insulin secretory pathway is largely unknown. Susceptibility of cells expressing ZnT-8 W325 to rapamycin but not to tacrolimus supports the tolerance of the W325 variant to calcineurin activity. Additional support was provided by the experiment using the DSCR1 construct. An endogenous calcineurin inhibitor, the DSCR1 C-terminal region (amino acids 134–197; DSCR1-CT) has been shown to bind to calcineurin and subsequently inhibit calcineurin activity.[27,28] The differential response pattern of the two ZnT-8 variants to DSCR1 provides strong support for the notion that the functional differences in ZnT-8 polymorphic variants are linked to their susceptibility to calcineurin.

This raises the question of why cells expressing ZnT-8 W325 showed resistance to CsA. One possibility is that ZnT-8 R325 may be a constituent of a phosphorylation motif for S327 (R-X-S/T) in the C terminus of ZnT-8. Dephosphorylation of the residue at 327 by calcineurin, which is phosphorylated under basal conditions, may lead to changes in ZnT-8 function, for example, to an increase in transporter activity, whereas the W325 variant may not allow the phosphorylation of S327 and hence may not be subjected to regulation by calcineurin. This hypothesis might explain why ZnT-8 W325 is protective only against calcineurin inhibitors.

Several studies have reported a higher incidence of PTDM in African-American populations than in Caucasian or Asian populations.[32,35,36] HapMap data show that the protective W allele frequency was much lower in the African group than in other ethnic populations (the W allele frequency was 25% in a group of Caucasian Americans with European ancestry, 48% in a Han Chinese population and 6% in a Yoruba population in Nigeria).[37] These data may also support the association of W325 with a low incidence of PTDM. A comparative study of insulin secretion responsiveness among patients from different racial populations receiving immunosuppressive drugs after kidney transplantation would provide further information about PTDM pathogenesis.

In conclusion, although definitive *in vivo* evidence is lacking, these data suggest that ZnT-8 might be an important molecule connecting calcineurin inhibition by CsA to downregulation of β-cell insulin secretion, and this signaling pathway might be a new therapeutic target for the treatment of CsA-induced β-cell complications that occur after organ transplantation.

Conflict of interest

The authors declare no conflict of interest

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