PRMT4 is involved in insulin secretion via the methylation of histone H3 in pancreatic β cells

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Abstract

The relationship between protein arginine methyltransferases (PRMTs) and insulin synthesis in β cells is not yet well understood. In the present study, we showed that PRMT4 expression was increased in INS-1 and HIT-T15 pancreatic β cells under high-glucose conditions. In addition, asymmetric dimethylation of Arg17 in histone H3 was significantly increased in both cell lines in the presence of glucose. The inhibition or knockdown of PRMT4 suppressed glucose-induced insulin gene expression in INS-1 cells by 81.6 and 79% respectively. Additionally, the overexpression of mutant PRMT4 also significantly repressed insulin gene expression. Consistently, insulin secretion induced in response to high levels of glucose was decreased by both PRMT4 inhibition and knockdown. Moreover, the inhibition of PRMT4 blocked high-glucose-induced insulin gene expression and insulin secretion in primary pancreatic islets. These results indicate that PRMT4 might be a key regulator of high-glucose-induced insulin secretion from pancreatic β cells via H3R17 methylation.

Key Words

- ▶ PRMT4
- ▶ insulin synthesis
- ▶ insulin secretion
- ▶ histone H3R17

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Introduction

Protein arginine methyltransferase 4 (PRMT4) is one of the nine subclasses of mammalian PRMTs, and it is a member of the type 1 PRMT family (Bedford & Clarke 2009). PRMT4 has been shown to catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the guanidino nitrogens of arginine residues to form ω -N^G,N^G-asymmetric dimethyl-arginine (aDMA; Cheng *et al.* 2007). PRMT4 has also been shown to catalyze the methylation of specific arginine residues in the N-terminal tails of histone H3, Arg2, Arg17, and Arg26 (Schurter *et al.* 2001). Two groups have reported that asymmetric dimethylation of Arg2 in histone H3 was mainly catalyzed by PRMT6 (Guccione *et al.*

2007, Iberg *et al.* 2008). Chromatin immunoprecipitation (ChIP) analysis revealed elevated arginine dimethylation of histone H3R17 at the promoters for pS2 (Bauer *et al.* 2002), E2F transcription factor 1 (Fietze *et al.* 2008), and cyclin E1 (CCNE1; El Messaoudi *et al.* 2006). The results of these studies indicate that PRMT4 plays a critical role in transcriptional regulation as a co-activator.

Insulin biosynthesis and secretion in pancreatic β cells play central roles in the control of glucose homeostasis in mammals. Insulin release is induced by several secretagogues, such as glucose (Grill *et al.* 1978), some amino acids (Newsholme & Krause 2012), glucagon-like peptide 1

(Irwin *et al.* 2007), and free fatty acids (Komatsu *et al.* 1999). Among these secretagogues, glucose is the most physiologically relevant, and it promotes insulin secretion by inducing multiple signaling pathways, including the triggering and amplifying pathways (Henquin 2000). The results of a previous study indicated that arginine methylation of the endogenous 20-kDa protein in HIT β cells was increased by glucose and was strongly correlated with insulin secretion in a glucose-dependent manner (Lim *et al.* 2003). In addition, the depletion of extracellular calcium by EGTA significantly reduced insulin secretion as well as arginine methylation of the protein. It was also reported that impaired enzymatic activity of PRMT1 and protein methylation were involved in postprandial hyperglycemia in Goto-Kakizaki (GK) rats (Iwasaki 2009), which indicates that protein methylation might be involved in insulin secretion.

In the present study, we investigated the relationship between PRMT-mediated histone arginine methylation and insulin secretion from pancreatic β cells. Among the type 1 PRMTs, the expression of PRMT4 was specifically increased by glucose and was correlated with histone H3R17 methylation during insulin gene expression and insulin secretion. Significantly, PRMT4 inhibition repressed insulin gene expression and secretion in pancreatic cells and in primary mouse pancreatic islets.

Materials and methods

Reagents

Anti-PRMT1, anti-PRMT4, anti-histone H3R17, anti-histone H3, and anti-hemagglutinin (HA) antibodies were purchased from Abcam (Cambridge, UK). Anti- β -actin antibody was purchased from Sigma. Anti-PRMT3 antibody was obtained from Millipore–Upstate (Billerica, MA, USA). Anti-PRMT6 antibody was obtained from IMGEX (San Diego, CA, USA). ECL reagent was purchased from Amersham. PRMT4 inhibitor was obtained from Merck Millipore (Billerica, MA, USA). pHM6-HA-PRMT4 plasmid was kindly provided by Dr Eui-Ju Choi (Korea University, Seoul, Korea).

Cell culture

Pancreatic β -cell line HIT-T15 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HIT-T15 cells were routinely grown in RPMI-1640 culture medium (Sigma) containing 10% fetal bovine serum (FBS), 0.8 mM glucose, 100 U/ml penicillin, and 100 μ g/ml

streptomycin in humidified air containing 5% CO₂ at 37 °C. INS-1 cells were grown in RPMI-1640 culture medium containing 10% FBS, 6 mM glucose, 50 μ mol/ml β -mercaptoethanol, 10 mmol/ml HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin in humidified air containing 5% CO₂ at 37 °C. Cells were subcultured 3–5 days after seeding.

Western blotting

β cells were washed three times with 1 $\times$ PBS and harvested with a scraper. After the cells were lysed on ice in lysis buffer (7 M urea, 2 M thiourea, and 65 mM dithiothreitol), the lysates were sonicated on ice. The cell extracts were then centrifuged at 12 000 *g* for 15 min, and the supernatants were collected. Thirty micrograms of protein were separated by 12% SDS–PAGE and then transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were incubated with the primary antibodies in blocking buffer (5% non-fat dry milk in 20 mM Tris–HCl and 150 mM NaCl (TBS) with 0.1% Tween-20 (TBST)) overnight at 4 °C. The immunoblots were rinsed three times in TBST and then incubated with the peroxidase-conjugated secondary antibody in TBST for 1 h at room temperature. Lastly, the membrane was rinsed three times with TBST for 10 min. Chemiluminescent detection was performed by using the Immobilon western chemiluminescent HRP substrate according to the manufacturer's instructions (Amersham Biosciences), and then the membrane was exposed to X-ray film.

siRNA transfection

ON-TARGET plus SMART pool siRNAs against rat PRMT4 (363026) and a non-targeting control were purchased from Dharmacon (Thermo Fisher Scientific Bioscience, Inc., Lafayette, CO, USA). INS-1 cells were seeded into six-well plates at a density of 2.0×10^6 cells/well before transfection. The PRMT4-specific siRNA (20 nM) and the non-specific siRNA (20 nM) were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were incubated with the transfection complexes for 4 h. After the medium containing the transfection reagents was aspirated, fresh medium was added. Cells were harvested 48 h after transfection for protein preparation.

Quantitative real-time PCR

Total RNA from INS-1 cells was extracted using QIAzol Lysis Reagent (Qiagen). The concentration of total RNA

was measured using a Nano Drop ND-100 device (Thermo Fisher Scientific). One microgram of mRNA isolated from INS-1 cells or C57BL/6 mouse islets was used for RT. First-strand cDNA was synthesized using quantitative real-time PCR. Real-time RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The primers used to amplify insulin were as follows: forward: 5'-CTT TTG TCA AAC AGC ACC T-3' and reverse: 5'-TAG TTC AGT TGG TAG AG-3'. The β -actin primers used were as follows: forward: 5'-GTC GTA CCA CTG GCA TTG TG-3' and reverse: 5'-CTC TCA GCT GTG GTG GTG AA-3'. The cycling conditions for the real-time PCR for all of the samples were as follows: an initial step for 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 15 s at 58 °C, and 1 min at 72 °C. The quantity of insulin mRNA was normalized to β -actin mRNA levels.

Islet preparation

Six-week-old male C57BL/6 mice were obtained from Central Lab Animal, Inc. (Seoul, Korea). All of the procedures used in the present study were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Korea University (KUIACUC-2014-267). Pancreatic islets were isolated from the C57BL/6 mice by collagenase digestion and Ficoll density gradient centrifugation (densities: 1.100, 1.085, 1.069, and 1.037; Source BioScience, Nottingham, UK). Two milliliters of Hank's buffered saline solution (HBSS) without FBS containing collagenase P (0.8 mg/ml; Roche Diagnostics) were injected into the common bile ducts of the mice. Tissues from seven to ten pancreases were transferred into 50 ml conical tubes and digested for 13 min and 20 s at 37 °C. Digestion was terminated by adding cold HBSS containing 5% FBS. The supernatant was discarded, and the tissue was resuspended in serum-free HBSS buffer. Digested tissue was filtered through an 85 ml stainless-steel strainer (40-mesh pore size; Sigma). The pellet was washed with serum-free HBSS three times. The islets were then separated by discontinuous Ficoll density gradient centrifugation at 800 *g* for 20 min at 4 °C. The islets were washed with HBSS and then pre-incubated in Krebs-Ringer bicarbonate-HEPES (KRBH) buffer for 1 h before being stimulated with 25 mM glucose for 1 h.

Insulin assays

Insulin secretion following glucose stimulation was assessed as follows: INS-1 cells were plated into six-well plates at a density of 2.0×10^6 cells/well and grown in RPMI-1640 medium for 3 days. Cells were washed three times

with KRBH buffer (109 mM NaCl, 4.6 mM KCl, 5 mM NaHCO₃, 2 mM CaCl₂, 1 mM MgCl₂, 1.15 mM Na₃HPO₄, 0.4 mM KH₂PO₄, 0.1 mM MgSO₄, and 20 mM HEPES, pH 7.4, with 0.1% BSA). The cells were then pre-incubated in the same buffer for 1 h at 37 °C. Next, the cells were washed two times with the same buffer and incubated in KRBH with 25 mM glucose for 0–1 h. The insulin secreted into the medium was quantified with a commercial kit (cat no. AKRIN-011T; Shibayagi, Gunma, Japan).

Insulin content

INS-1 cells were seeded into six-well plates at a density of 1.5×10^6 cells/well before transfection. The pHM6-PRMT4 plasmids, which contained either WT or mutant PRMT4, were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After the medium containing the transfection reagents was aspirated, fresh medium was added. Cells were harvested 48 h after transfection for insulin preparation. Cells were washed three times with KRBH buffer. The cells were then pre-incubated in the same buffer for 1 h at 37 °C. Next, the cells were washed two times with the same buffer and incubated in KRBH with 25 mM glucose for 1 h. The extracts were sonicated on ice and incubated in an acid ethanol solution (0.18 M HCl, 96% ethanol, and 2.5% H₂O) overnight at 4 °C. The insulin content was quantified with a commercial kit (cat no. AKRIN-011T; Shibayagi).

Statistical analysis

All of the results are presented as means $\pm$ s.d. Student's *t*-test was used to determine the statistical significance of differences. All experiments were performed at least three times.

Results

PRMT4 expression is increased under high-glucose conditions in pancreatic β cells

To examine whether glucose alters the expression pattern of the endogenous PRMTs, two pancreatic cell lines, INS-1 and HIT-T15, were incubated in 25 or 16.7 mM glucose. Results of western blotting analysis using specific antibodies to the various PRMTs indicated that the level of PRMT4 was markedly increased in both cell lines (Fig. 1A and B). In contrast, the expression of other type 1 PRMTs (PRMT1, 3, and 6) was not affected by glucose stimulation. In addition, the expression of the type 2 PRMTs (PRMT5 and 7)

Figure 1

PRMT4 expression is increased under high-glucose conditions in pancreatic β -cells. (A) INS-1 β cells were pre-incubated in KRBH buffer without glucose for 1 h and then stimulated for the indicated time periods with 25 mM glucose in KRBH buffer. (B) HIT-T15 β cells were pre-incubated in KRBH buffer without glucose for 1 h and then stimulated for the indicated time periods with 16.7 mM glucose in KRBH buffer. Whole-cell lysates were

prepared as described in the 'Materials and methods' section. Thirty micrograms of the whole-cell lysates were subjected to 12% SDS-PAGE and western blotting with anti-PRMT antibodies. An anti- β -actin antibody was used as a loading control. The relative intensities of the detected proteins were quantified with a densitometer. * $P < 0.05$ versus basal conditions.

was also not changed (data not shown). To validate glucose-stimulated insulin secretion in INS-1 and HIT-T15 cells, the cells were continuously cultured in 6 or 0.8 mM glucose rather than with a high glucose concentration (11.1 mM; Robertson *et al.* 1992, Moran *et al.* 1997, Tanaka *et al.* 1999). As shown in Supplementary Figure 1, see section on supplementary data given at the end of this article, when INS-1 and HIT-T15 cells were stimulated with the indicated concentrations of glucose (25 or 16.7 mM), glucose-stimulated insulin secretion was increased in a time-dependent manner in the two pancreatic β cell-lines. These results suggest that the increase in PRMT4 expression might be associated with glucose-stimulated physiological events in β cells.

PRMT4 is involved in histone H3R17 methylation

To investigate the relationship between PRMT4 expression and histone H3R17 methylation following glucose stimulation in β cells, we used an anti-histone H3R17 antibody that specifically recognizes aDMA at this position. As shown in Fig. 2A and B, when INS-1 and HIT-T15 cells were incubated with 25 or 16.7 mM glucose, the asymmetric dimethylation of Arg17 in histone H3 was significantly increased. It has been reported that Arg17 and Arg26 methylation of histone H3 was associated with transcriptional activation (Bauer *et al.* 2002). We investigated whether the Arg26 methylation of histone H3 was changed by the indicated concentration of glucose (16.7 mM) for 2 h in HIT-T15 cells. As shown in Supplementary Figure 2, see section on supplementary data given at the end of this article, H3R26 methylation also increased in a time-dependent manner in HIT-T15 β cells.

To determine whether PRMT4 affects this histone arginine methylation (H3R17) in INS-1 cells following glucose stimulation, the cells were pretreated with a

PRMT4-specific inhibitor (7 μ M). As shown in Fig. 2C, after treatment with the PRMT4-specific inhibitor, the level of H3R17 methylation was significantly decreased. Similarly, the knockdown of PRMT4 with siRNA also blocked glucose-mediated H3R17 methylation, whereas the transfection of a non-targeting control siRNA did not affect the methylation (Fig. 2D). In addition, we transfected a plasmid carrying mutant PRMT4 to determine whether the enzyme activity of PRMT4 affects H3R17 methylation. As shown in Fig. 2E, the level of H3R17 methylation was decreased in cells transfected with the mutant PRMT4. These results indicate that PRMT4 is involved in high-glucose-mediated histone arginine methylation.

PRMT4 affects high-glucose-induced insulin gene expression

To investigate the relationship between insulin gene expression and histone H3R17 methylation in β cells, we pretreated INS-1 cells with a PRMT4-specific inhibitor (7 μ M) and analyzed insulin mRNA levels by quantitative real-time PCR analysis. After 30 min of pretreatment with PRMT4 inhibitor, the cells were stimulated with 25 mM glucose for 1 h. Figure 3A shows that the increase in the glucose-mediated expression of insulin was attenuated by PRMT4 inhibition. Next, we attempted to determine whether PRMT4 knockdown affected insulin mRNA levels. As shown in Fig. 3B, the level of insulin mRNA was reduced by PRMT4 siRNA transfection. We further examined whether PRMT4 enzyme activity affected insulin gene expression during glucose stimulation in INS-1 cells. As shown in Fig. 3C, the level of insulin mRNA was elevated 2.23-fold in cells that had been transfected with a WT PRMT4 as compared with control cells. However, the expression of insulin mRNA was repressed

Figure 2

PRMT4 is involved in histone H3R17 methylation. (A) INS-1 β cells were pre-incubated in KRBH buffer without glucose for 1 h and then stimulated with 25 mM glucose for 1 h. (B) HIT-T15 β cells were pre-incubated in KRBH buffer without glucose for 1 h and then stimulated with 16.7 mM glucose for 1 h. Histone H3R17 methylation was evaluated by western blotting with an anti-histone H3R17 antibody. An anti-histone H3 antibody was used as a loading control. The relative intensities of the detected proteins were quantified using a densitometer. (C) INS-1 cells were pretreated with a PRMT4 inhibitor (7 μ M) for 30 min during pre-incubation in KRBH buffer and then stimulated with 25 mM glucose for 1 h. Cell lysates (30 μ g) were analyzed by western

blotting with an anti-histone H3R17 antibody. An anti-histone H3 antibody was used as a loading control. (D) INS-1 cells were transfected with PRMT4 siRNA (20 nM) for 48 h. Cell lysates (30 μ g) were analyzed by western blotting with anti-PRMT4 and anti-histone H3R17 antibodies. Anti-histone H3 and anti- β -actin antibodies were used as loading controls. (E) INS-1 cells were transfected with a pHM6-PRMT4 plasmid containing mutant PRMT4 for 48 h. Cell lysates (30 μ g) were analyzed by western blotting with anti-PRMT4, anti-HA, and anti-histone H3R17 antibodies. Anti-histone H3 and anti- β -actin antibodies were used as loading controls. The error bars indicate the s.d. of triplicate measurements. * $P < 0.05$ versus basal conditions.

significantly in cells that were carrying a mutant PRMT4 overexpression plasmid. Together, these results indicate that PRMT4-mediated histone arginine methylation (H3R17) is involved in glucose-stimulated insulin transcription in INS-1 cells.

PRMT4 is involved in high-glucose-mediated insulin secretion

To investigate whether PRMT4 contributes to insulin secretion during glucose stimulation, we pretreated INS-1 cells with PRMT4 inhibitor (7 μ M) for 30 min before stimulating the cells with 25 mM glucose. As shown in Fig. 4A, glucose-induced insulin release was 2.27-fold higher than the basal level. However, PRMT4 inhibitor

pretreatment dramatically decreased insulin secretion. It has been demonstrated that insulin release by glucose stimulation is biphasic in pattern in pancreatic β cells (Straub & Sharp 2002). To confirm the effect of a short-term glucose treatment on insulin secretion, we measured insulin secretion at 20 min under 25 mM glucose in INS-1 cells. As shown in Supplementary Figure 3, see section on supplementary data given at the end of this article, glucose-stimulated insulin secretion was 2.01-fold higher than the control level. Moreover, pretreatment with PRMT4 inhibitor significantly decreased insulin secretion. This result indicated that the pattern of insulin secretion during short-term treatment (20 min) was not different from that during long-term treatment (60 min). To further explore the relationship between PRMT4 and insulin

Figure 3

PRMT4 affects insulin gene expression levels. (A) INS-1 cells were pretreated with a PRMT4 inhibitor (7 μ M) for 30 min during pre-incubation in KRBH buffer and then stimulated with 25 mM glucose for 1 h. (B) INS-1 cells were transfected with PRMT4 siRNA (20 nM) or a non-targeting siRNA (20 nM) for 48 h. (C) INS-1 cells were transfected with a pHM6-PRMT4 plasmid containing either WT or mutant PRMT4 for 48 h. Total RNA was extracted, and the insulin mRNA levels were analyzed by real-time PCR and normalized to endogenous β -actin expression levels. The error bars indicate the s.d. of triplicate measurements. * $P < 0.05$ versus basal conditions. ** $P < 0.05$ as compared with WT PRMT4 overexpression.

secretion, INS-1 cells were transfected with PRMT4 siRNA. As shown in Fig. 4B, transfection with PRMT4 siRNA also decreased insulin secretion as compared with that under the glucose-stimulated conditions, whereas transfection with a non-targeting siRNA had no effect on insulin secretion. We examined whether the activity of PRMT4 affected the insulin content of β cells by expressing WT and mutant PRMT4 and measuring insulin content. The cellular insulin content was determined as described previously (Andersson & Sandler 2001, Hamid *et al.* 2002). As shown in Fig. 4C, the insulin content of the controls was 175.2 ± 7.81 ng/ 10^6 cells. The insulin content under high-glucose conditions was not altered significantly by PRMT4 manipulation. These results indicate that the inhibition of PRMT4 exerts a negative effect on insulin secretion but does not affect insulin content in pancreatic β cells.

PRMT4 is involved in high-glucose-mediated insulin secretion in primary cultured islets

To corroborate the role of PRMT4 in glucose-induced insulin mRNA expression, we assessed the effect of PRMT4 inhibitor in primary islets. As shown in Fig. 5A, pancreatic islets from C57BL/6 mice were isolated, and when their morphology was assessed under a microscope, a golden brown color was observed (Carter *et al.* 2009). Pretreatment with PRMT4 inhibitor significantly downregulated (by 7.4%) the insulin gene expression induced under high-glucose conditions (Fig. 5B). In addition, insulin release in the presence of PRMT4 inhibitor was significantly suppressed as compared with that released from control islets (Fig. 5C). Collectively, these results indicate that PRMT4 might be a new potent transcriptional co-activator of glucose-induced insulin gene expression in pancreatic β cells.

Discussion

In the present study, we showed that the incubation of pancreatic β cells in medium with high levels of glucose increased the expression of PRMT4. We also demonstrated that PRMT4-mediated increased histone H3R17 methylation was correlated with insulin gene expression and secretion during glucose stimulation in pancreatic β cells.

Insulin is a peptide hormone secreted by the β cells of the pancreas. Insulin is synthesized in response to several stimuli, including the ingestion of protein and the presence of glucose in the blood. Endocrine protein accounts for only 2–3% of the total pancreatic mass. Within the islets, β cells constitute about 70% of the cells.

Figure 4

PRMT4 is involved in high-glucose-mediated insulin secretion. (A) INS-1 cells were pretreated with KRBH buffer without glucose for 1 h and with PRMT4 inhibitor (7 μ M) for 30 min. Cells were stimulated with 25 mM glucose in KRBH buffer for 1 h. (B) Cells were transfected with PRMT4 siRNA (20 nM) or a non-targeting siRNA (20 nM) for 48 h. Then, these cells were stimulated with 25 mM glucose in KRBH buffer for 1 h. After incubation, aliquots were withdrawn from the culture medium and determined using an insulin antiserum immunoassay. Error bars indicate the s.d. of triplicate measurements. * $P < 0.05$ versus basal conditions or glucose-stimulated conditions. (C) INS-1 cells were transfected with a pHM6-PRMT4 plasmid containing either WT or mutant PRMT4 for 48 h. Cells were pre-incubated in KRBH buffer without glucose for 1 h and then stimulated with 25 mM glucose for 1 h. After incubation, the extracts were sonicated on ice and incubated in an acid ethanol solution (0.18 M HCl, 96% ethanol, and 2.5% H₂O) overnight at 4 °C. The insulin content was determined using an insulin antiserum immunoassay. Error bars indicate the s.d. of triplicate measurements. (mean $\pm$ s.e.m., $n = 4$).

Insulin is regulated by controlling the activity of the insulin promoter. Many transcription factors bind to the insulin promoter. For example, pancreatic and duodenal homeobox 1 (Pdx1) binds to the A-boxes, NeuroD binds to the E-boxes, the mammalian homologue of avian MafA/l-Maf (MafA) binds to the C-boxes, and cAMP response element binding proteins bind to cAMP response elements. It has been reported that Pdx1 links histone methylation to insulin gene transcription (Francis *et al.* 2005). These authors proposed a model wherein an epigenetic mechanism that involved histone methylation played a critical role in the regulation of insulin gene transcription. In the present study, our results indicated that histone methylation was controlled by PRMT4 expression. The expression of PRMT4 was specifically affected by high-glucose conditions. Therefore, we speculate that histone methylation may be involved in insulin gene transcription under high-glucose conditions.

It is very well known that a higher-order chromatin structure influences transcriptional regulation. In eukaryotes, histone H2A, H2B, H3, and H4 are the proteins that comprise the chromatin core unit. Histone modification alters chromatin structure, which in turn alters gene transcription. Hyperacetylation of H3 and H4 is correlated with transcriptionally active genes, and hypoacetylation of H3 and H4 is associated with transcriptionally inactive genes (Chakrabarti *et al.* 2003, Schneider *et al.* 2004). It has been demonstrated that the insulin promoter has highly dimethylated H3K4 (Chakrabarti *et al.* 2003, Francis *et al.* 2005), which indicates that histone methylation may be associated with insulin promoter regulation.

PRMT4 has been shown to methylate arginine residues (R17 and R26) in the N-terminal region of histone H3. Moreover, these residues have been proposed to be correlated with transcriptional activation (El Messaoudi *et al.* 2006, Wu *et al.* 2009). Recently, the methylation of R42 in histone H3 by PRMT4 was associated with transcriptional activation of the target gene (Casadio *et al.* 2013). Among these arginine residues, H3R17 is well known as the major site for gene expression (Ma *et al.* 2001, Daujat *et al.* 2002, An *et al.* 2004). Because we did not confirm that H3R26 methylation is involved in insulin gene expression, further studies are needed to determine the relationship between insulin gene expression and these modifications at insulin promoter sites by ChIP assay.

In the present study, PRMT4 was induced under high-glucose conditions. The inhibition of PRMT4 blocked high-glucose-induced insulin transcription and secretion, which indicates that PRMT4 is involved in insulin gene regulation. We did not determine which transcription

Figure 5

PRMT4 is involved in high-glucose-mediated insulin secretion in primary cultured islets. (A) Freshly isolated pancreatic islets from C57BL/6 mice ($n=14$) were pre-incubated with KRBH buffer in the absence of glucose for 1 h and then incubated with PRMT4 inhibitor ($7 \mu\text{M}$). Islets were stimulated with 25 mM glucose in KRBH buffer for 1 h. Pancreatic islets were isolated and observed under a microscope. (B) Islets were pre-incubated with KRBH buffer without glucose for 1 h and then incubated with PRMT4 inhibitor ($7 \mu\text{M}$). Islets were stimulated with 25 mM glucose in KRBH buffer for 1 h. Total RNA was extracted, and the mRNA levels of the insulin gene were analyzed by real-time PCR and normalized to endogenous β -actin expression levels. The error bars indicate the s.d. of triplicate measurements. (C) Islets were pre-incubated with KRBH buffer without glucose for 1 h and then incubated with PRMT4 inhibitor ($7 \mu\text{M}$). Islets were stimulated with 25 mM glucose in KRBH buffer for 1 h. After incubation, aliquots of the KRBH buffer were withdrawn and subjected to insulin antiserum immunoassay. The error bars indicate the s.d. of triplicate measurements. * $P<0.05$ versus basal conditions or glucose-stimulated conditions.

factors are affected by PRMT4; therefore, we could not define the signaling pathways of the high-glucose–PRMT4–histone arginine methylation–insulin axis. In the future, the transcription factors involved should be clarified.

According to the results of previous studies, the two cell lines INS-1 and HIT-T15 are different in many ways. First, INS-1 cells display a relatively high insulin content as compared with other cell lines (Hamid *et al.* 2002). Many papers have been published reporting that 25 mM glucose is the optimal concentration for insulin gene transcription in INS-1 cells (Webb *et al.* 2000, Ort *et al.* 2001). Second, HIT-T15 cells have been shown to be responsive to glucose and are appropriate models for studying insulin secretion (Hill & Boyd 1985). These cells have displayed an elevation in insulin gene transcription with glucose stimulation (16.7 mM; Leibiger *et al.* 1998). We therefore speculated that the optimal glucose levels for insulin secretion or gene expression in each cell were different. We consequently used 25 mM glucose in INS-1 cells and 16.7 mM in HIT-T15 cells.

It has been reported that synaptotagmin 7 (Syt VII) is a Ca^{2+} -dependent regulator of exocytosis. Pancreatic islets isolated from Syt VII-null mice have been shown to have attenuated insulin secretion (Li *et al.* 2007). In addition, HnRNP R, which is a protein that interacts with Syt VII, is a known PRMT1 substrate (Mizutani *et al.* 2000, Wada *et al.* 2002). We showed that the amount of PRMT4 expression under high-glucose conditions was correlated with the amount of insulin secretion. However, we did not determine the molecular pathways of protein arginine methylation and insulin secretion in β cells. It has been reported that the arginine methylation of 66-kDa protein was significantly reduced and correlated with the attenuation of insulin secretion in the pancreases of GK rats (Iwasaki 2009). Collectively, it is possible that the contribution of PRMT4 to insulin secretion in β cells may be mediated by complicated indirect mechanisms.

The release of insulin by glucose is biphasic in pattern in pancreatic β cells. The initial insulin secretion in response to glucose (first phase) was associated with 0.4% of the total granules (about 13 000) in the cell. Following the initial insulin secretion, the sustained second phase was then released from the reserve pool within 5–60 min, and the rate of release of granules was small (Dean 1973, Straub & Sharp 2002). It has also been demonstrated that the insulin synthesis rates under high-glucose conditions were controlled by multiple mechanisms, such as gene transcription, mRNA splicing, and translation (Shalev *et al.* 2002). To restore the newly synthesized insulin from gene expression to mature

granules, prolonged glucose stimulation for more than 12 h is required (Ren *et al.* 2007). In the present study, the alteration of insulin content by PRMT4 manipulation in β cells was not significant under high-glucose stimulation for 1 h. Therefore, the alteration of insulin content may not be influenced by glucose stimulation for 1 h.

Under long-term high-glucose conditions, pancreatic β cells cause the accumulation of reactive oxygen species (ROS), which might cause dysfunction in β cells. For example, the expression of insulin mRNA and the binding ability of transcription factors to the insulin promoter have been shown to progressively and dramatically decrease in β cells (Robertson *et al.* 1992). Abnormal ROS levels reduce insulin gene expression, glucose-stimulated insulin secretion, and insulin content. It has also been reported that an increase in PRMT1 expression and elevated asymmetric dimethylarginine are associated with ROS-mediated diabetic retinopathy (Chen *et al.* 2009). These results indicate that there might be a relationship between ROS and PRMT; thus, further study is required to characterize the molecular role of PRMT4 in β -cell dysfunction.

In summary, the interplay between histone arginine methylation and insulin secretion and/or synthesis induced by glucose in β cells is not yet well understood. Pancreatic islets obtained from an animal model with type 2 diabetes are characterized by β -cell dysfunction, which impairs insulin secretion. In the present study, we investigated the relationship between PRMT expression and the alteration in insulin gene expression in β cells. We identified PRMT4 as a key factor for high-glucose-mediated histone methylation. These findings emphasize the necessity and importance of using microarrays in future studies to determine which genes are regulated by PRMT4.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0325>.

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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