Protein Arginine Methyltransferase 4 Regulates Adipose Tissue Lipolysis in Type 1 Diabetic Mice

Purpose: Hypertriglyceridemia is considered to be driven by increased lipolysis in type 1 diabetes mellitus (T1DM). However, information regarding the transcriptional circuitry that governs lipolysis remains incomplete in T1DM. Protein arginine methyltransferase 4 (PRMT4), a transcriptional coactivation factor, promotes autophagy and may play an important role in lipolysis. We wonder whether activated lipolysis in T1DM is regulated by PRMT4.

Materials and Methods: Recombinant adeno-associated virus was adopted to overexpress PRMT4 in adipose tissue of mice. Streptozotocin (150 mg/kg) was injected intraperitoneally into mice to induce T1DM. Plasma insulin, triglycerides, free fatty acids (FFAs) levels were determined using commercial assay kits. Differentiated adipocytes were applied to verify the regulation of PRMT4 on lipolysis.

Results: Elevated serum triglycerides and FFAs were observed in PRMT4-overexpressed T1DM mice. We also observed that PRMT4 over-expression induced the decrease of fat pads weights and adipocyte sizes. Moreover, expression levels of lipolysis-related molecules, including ATGL, HSL, and MAGL, and HSL phosphorylation levels were increased in PRMT4-overexpressed mice when compared to those of control mice. In vitro, PRMT4 promoted FFAs release and activated HSL phosphorylation, whereas PRMT4 knockdown inhibited these processes.

Conclusion: PRMT4 promotes lipolysis and increases serum triglyceride in T1DM.

Keywords: type 1 diabetic mice, hypertriglyceridemia, protein arginine methyltransferase 4, lipolysis

Introduction

The prevalence of dyslipidemia is much higher in patients with type 1 diabetes mellitus (T1DM), especially in children and youth with the disease. Diabetic dyslipidemia is characterized by a decreased concentration of high-density lipoprotein–cholesterol, increased low-density lipoprotein–cholesterol, and elevated concentration of plasma triglycerides. In T1DM, the triglyceride levels are increased and are associated closely with the risk of cardiovascular diseases. It has been suggested that hyperlipidemia is induced by the increase triglyceride secretion attributed to liver lipid synthesis driven by sterol regulatory element-binding protein 2 (SREBP2) in type 2 diabetes mellitus (T2DM). However, liver lipid synthesis is not increased in T1DM owing to the lack of insulin, which is the activator of SREBP2. Therefore, increased lipid synthesis in the liver is not the cause of hyperlipidemia in T1DM. In fact, it is the insulin deficiency that leads to increased lipolysis and elevated levels of plasma triglycerides in T1DM.
Lipolysis involves the hydrolysis of triglycerides and the release of free fatty acids (FFAs). Increased flux of FFAs promotes triglyceride production in the liver,\(^8\) thus leads to elevated triglyceride secretion and higher triglyceride concentrations.\(^9\) Lipolysis consists of the sequential hydrolysis of triglycerides, which was catalyzed by three different enzymes: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase ( MAGL).\(^10\) It has been suggested that lipolysis is induced by catecholamines or other agonists, and is suppressed by insulin.\(^11\) Mechanistically, insulin inhibits adipocyte lipolysis via mTORC1-Egr1-ATGL-mediated pathway.\(^12\) However, the regulation mechanism was not confirmed in diabetic mice. The transcriptional circuitry through which insulin governs lipolysis in T1DM remains unclear. Given the consequences of the lipolysis and dyslipidemia in T1DM, understanding the mechanism underlying the regulation of lipolysis in the adipocytes is of considerable importance.

Protein arginine methyltransferase 4 (PRMT4), also known as coactivator-associated arginine methyltransferase 1 (CARM1), converts the monomethylarginine intermediate to asymmetric dimethylarginine. PRMT4 is widely expressed in tissues throughout the body under physiological conditions and has been confirmed to be highly expressed in cancers.\(^14\) In patients with T2DM, PRMT4 is overexpressed in the white blood cells.\(^15\) A previous study has shown that this enzyme is also overexpressed in retinal pigment epithelial cells of rats with T1DM, where it induces apoptosis in these cells.\(^16,17\) PRMT4 regulates various cellular processes, such as DNA repair, transcriptional coactivation, mRNA splicing, and protein translocation.\(^18-22\) It has also been suggested that PRMT4 functionally implicated in glycogen metabolism\(^23,24\) and adipocyte differentiation.\(^25\) PRMT4 also plays a crucial role in autophagy after nutrient starvation.\(^26\) Meanwhile, the upregulation of autophagy promotes fat mobilization and lipolysis.\(^27\) Based on previous research, we speculated that PRMT4 might be related to lipolysis. Therefore, the purpose of our study was to explore whether PRMT4 be required for activated lipolysis and hypertriglyceridemia in T1DM.

In this study, the expression of PRMT4 will be explored in the white adipose tissue (WAT) of mice with streptozotocin (STZ)-induced diabetic mice. To determine the effect of PRMT4 on adipose function, its role in lipolysis will be investigated using mouse models of T1DM and fasted mice in vivo and mouse primary adipocytes in vitro.

### Materials and Methods

#### Animal Experiments and Recombinant Adeno-Associated Virus Transduction

Wild-type C57BL/6 mice (male, 6-weeks old) were purchased from SPF Biotechnology Ltd (Beijing, China). All the animal experiments were approved by the Ethical Committee of Huazhong University of Science and Technology and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The mice were housed in a specific pathogen-free room with a controlled light/dark cycle (lights on 08:00–20:00).

Recombinant adeno-associated virus (AAV) vectors of serotype 9 harboring the murine PRMT4 gene were generated by replacing the mCherry fragment in the AAV cloning vector pAOV-CMV-mCherry with PRMT4-3X Flag.\(^28\) The vector pAOV-CMV-mCherry was used to produce empty vectors for transduction into control mice.

T1DM was induced in 6-weeks old male mice by injection of STZ dissolved in 100 mmol/L citrate buffer as described previously\(^29\) with some modifications. Briefly, mice were fasted overnight and then injected intraperitoneally with a dose of STZ (150 mg/kg). One week after intraperitoneal injection, mice were monitored for hyperglycemia on days 7, 10, and 14. Only mice in which random blood glucose exceeded 16.7 mM for the following week were assigned to the diabetic group.

#### White Adipose Tissue Morphology Analysis

The tissues isolated from T1DM mice were dehydrated and embedded, and 4-µm sections were stained with hematoxylin and eosin (H&E). The adipocyte size was determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

#### Glucose, Insulin, and Lipid Determination

The blood glucose level of each mouse was measured using a glucose meter (OneTouch Ultravue, Johnson & Johnson Medical Devices Companies, New Brunswick, NJ, USA). The serum insulin, triglyceride, and FFA levels were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturers’ instructions.

#### Stromal Vascular Fraction Isolation

Primary stromal vascular fraction (SVF) was isolated as described previously\(^30\) with some modifications. Briefly, wild-type C57BL/6 mice were sacrificed and the adipose
tissues were processed to obtain stromal vascular fractions (SVFs). After digestion in Dulbecco’s modified Eagle’s medium (DMEM) containing 1% collagenase type II at 37°C for 1 h, the mixture was filtered and centrifuged to obtain the SVF-containing pellet.

**Preadipocyte Differentiation and Adenoviral Transduction**

Preadipocytes differentiation and adenovirus infection were carried out as described previously with some modifications. Briefly, 2 days after confluence, the SVF cells were treated with insulin (167 nM), 3-isobutyl-1-methylxanthine (500 μM), rosiglitazone (2.5 μM), and dexamethasone (2 μM) for 2 days. Next, the cells were cultured in DMEM containing insulin (167 nM), and rosiglitazone (2.5 μM) for 6 days.

The differentiation adipocytes were infected with the adenovirus vector carrying the coding sequence of PRMT4 (Ad-PRMT4). Meanwhile, an adenovirus vector containing the gene for the green fluorescence protein only was applied as a negative control (Ad-ctrl). To knock down the expression of PRMT4 in adipocytes, adenovirus expressing a short hairpin RNA (shRNA) against PRMT4 (sh-PRMT4) was used. Cells infected with adenovirus expressing the universal control shRNA (sh-NC) were used as control.

**Lipolysis Assay in Adipocytes**

Differentiated adipocytes were infected for 48 h with Ad-PRMT4 or Ad-sh-PRMT4 and then incubated for 2 h in culture media containing 2% fatty acid-free bovine serum albumin (BSA) in the presence or absence of 1 mM CL316, 243 or 10 nM insulin. Lipolysis was assessed by the release of the FFA content.

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR**

Total RNA was isolated and reverse transcribed as described previously with some modifications using commercial kits (Takara Biological Incorporated, Shiga, Japan). The mRNA levels were tested by qPCR using the SYBR Green I Master Mix (Takara Biological Incorporated, Shiga, Japan). The primers applied to real-time qPCR were as follows:

STZ-induced Diabetes

Increased Serum Triglycerides in Mice with STZ-Induced Diabetes

PRMT4 Was Upregulated in White Adipose Tissue and Associated with the Activation of Lipid Metabolic Signaling Pathways

To investigate whether this enzyme is upregulated in adipose tissue, we tested the protein expression of PRMT4 in epididymal white adipose tissue (eWAT) from diabetic mice and found that its expression was more abundant compared to control mice.
with that in non-diabetic mice (Figure 1A and B). To further evaluate the roles of PRMT4 in eWAT, the enzyme was overexpressed in the WAT of diabetic mice via tail vein injection with recombinant AAV carrying the enzyme-coding gene. Consequently, the serum triglycerides were increased by 42% and FFAs were increased by 26% in the diabetic mice infected with the PRMT4-carrying AAV (PRMT4-STZ group) relative to that of mice infected with the AAV control (Ctrl-STZ group), as shown in Figure 1C and D. While, PRMT4 had no effect on the mouse body weight (Figure 1E) and food intake (Figure 1F), and the blood glucose and serum insulin levels were not changed in the PRMT4-overexpressing mice (Figure 1G and H). PRMT4 Overexpression Promotes Lipolysis in Type 1 Diabetic Mice and in Fasted Mice

A previous study has shown that lipolysis was the primary modulator of triglyceride levels in mice with STZ-induced diabetes. Therefore, we further explored the role of PRMT4 in lipolysis and tested the indicators that reflect fat breakdown. We found that PRMT4 overexpression in diabetic mice resulted in a significant reduction of fat pads weights (Figure 2A and B). Next, we examined the size of the adipocytes in WAT. As shown in Figure 2C and D, the adipocyte size of the PRMT4-STZ group mice was smaller than that in the Ctrl-STZ group.
Since fasted mice are considered to be classical models for studying insulin deficiency lipolysis, we explored the effect of PRMT4 on lipolysis in these mice. The mice were deprived of food from 8 am to the following 8 am with free access to drinking water. A reduction in fat pads (Figure 2E) and a significant increase in the serum FFAs (Figure 2F) were observed in the mice overexpressing PRMT4 (PRMT4-Fasted) compared with that in the control mice (Ctrl-Fasted). Lipolysis was enhanced by PRMT4 overexpression in fasted mice.

**Figure 2 PRMT4 overexpression promotes lipolysis in mice.**

Notes: (A–D) Lipolysis was tested in mice with streptozotocin (STZ)-induced diabetes and in control mice. The gross morphology of the fat pads (A) and the tissue weights (B) of the inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) were recorded. Representative images of H&E-stained sections of eWAT (C), and frequency distribution of the adipocyte sizes of eWAT (D). (E and F) Weights of the iWAT and eWAT (E) and levels of serum-free fatty acids (FFAs) (F) determined in adeno-associated virus (AAV) control-infected fed mice (Ctrl-Fed), AAV-control-infected fasted mice (Ctrl-Fasted), and AAV-PRMT4-infected fasted mice (PRMT4-Fasted).

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**PRMT4 Upregulates the Lipolysis-Related Pathway in Adipose Tissues**

To test the hypothesis that PRMT4 affects lipolysis, we investigated the lipolysis pathway, which is regulated by multiple processes and molecules. qPCR analysis revealed that the mRNA levels of ATGL, HSL, and MAGL in adipocytes isolated from WAT of the PRMT4-STZ mice were elevated relative to that of the Ctrl-STZ mice (Figure 3A). Additionally, the HSL activity, which was measured by HSL phosphorylation at Ser660, increased in the PRMT4-overexpressing adipose tissue (Figure 3B and C).

The lipolysis pathway was also evaluated in fasted mice. Consistent with the results in the diabetic mice, the mRNA levels of ATGL, HSL, and MAGL in adipocytes isolated from WAT of the PRMT4-STZ mice were elevated relative to that of the Ctrl-STZ mice (Figure 3D). Additionally, HSL phosphorylation at Ser660 increased in the adipose tissue of PRMT4-overexpressed mice (Figure 3E and F).
To explore the role of PRMT4 on lipolysis in vitro, we generated PRMT4-overexpressing adipocyte models by infecting adipocytes differentiated from SVFs with the Ad-PRMT4 or Ad-ctrl vectors. After 48 h of infection, the cells were incubated in media with the presence or absence of the β3-adrenergic receptor agonist CL316,243 and/or insulin. As revealed in Figure 4A, CL316,243 significantly stimulated FFAs release and this effect was inhibited by insulin administration. Compared with that in the control cells, the amount of FFAs released—an indicator of lipolysis and induced by CL316,243—increased in the PRMT4-overexpressing cells. Meanwhile, the inhibitory effect of insulin on the CL316,243-induced activation of HSL phosphorylation was absent (Figure 4E and F).

Additionally, HSL phosphorylation at Ser660 in the adipocytes was examined by Western blot assay. Consistent with the FFA release amount, the HSL phosphorylation level was elevated in the PRMT4-overexpressing adipocytes (Figure 4C and D). Moreover, the inhibitory effect of insulin on the CL316,243-induced activation of HSL phosphorylation was absent (Figure 4E and F).

**Discussion**

In this study, we showed that PRMT4 induced lipolysis and increased the serum triglycerides in diabetic mice. Two different model systems were applied in our study; namely, T1DM mice, and fasted mice. The data showed that PRMT4 overexpression was accompanied by hyperactive lipolysis in both the diabetic and fasted mice, which was also verified by its promotion of CL316,243-induced lipolysis in mouse primary adipocytes. In summary, the upregulation of PRMT4 in WAT resulted in hyperactive lipolysis and a marked increase in the serum triglyceride levels in T1DM.

PRMT4 is widely expressed in tissues throughout the body and overexpressed in human patients with cancers and T2DM.14,15 In our study, increased PRMT4 expression was
observed in the WAT of STZ-induced mice. However, PRMT4 expression varies among different tissues in STZ-induced models of diabetes. For example, its expression increased in the retinal pigment epithelial layer, whereas it diminished in the glomerular epithelium of rats with STZ-induced diabetes. The factors that upregulate the level of PRMT4 in WAT of T1DM mice remain unclear. Previous studies had clarified that PRMT4 expression was upregulated by high glucose levels in retinal pigment epithelial cells, and by insulin in hepatocytes. On the basis of our present analysis, we infer that the upregulation of PRMT4 in the adipose tissue of diabetic mice may be caused by hyperglycemia rather than insulin, the latter of which is deficient in diabetic mice.

Since PRMT4 has been observed to be upregulated in the WAT of STZ-treated mice, what is the role of this enzyme plays in adipocyte function? PRMT4 promotes adipocytes differentiation and lipid synthesis in vitro by coactivating peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein beta (C/EBPβ). However, the effect of PRMT4 on lipid synthesis in adipocytes has not been confirmed in vivo. As suggested in our experiments, PRMT4 overexpression activated lipolysis in STZ-induced diabetic mice and fasted mice and promoted

Figure 4 PRMT4 regulates lipolysis in mouse primary adipocytes.
Notes: (A) Free fatty acids (FFAs) levels in culture media of primary adipocytes infected with adenovirus carrying PRMT4 (Ad-PRMT4) or control adenovirus (Ad-ctrl). (B) FFAs levels in culture media of primary adipocytes infected with adenovirus carrying sh-PRMT4 (sh-PRMT4) or adenovirus carrying the negative control (sh-NC). Cells were treated or not treated with 1 μM CL316,243 and/or 10 nM insulin for 2 h. (C and D) Western blot results of HSL phosphorylation at Ser660 in adipocytes infected with Ad-PRMT4 or Ad-ctrl after treatment as described in part (A). (E and F) Western blot results of HSL phosphorylation at Ser660 in adipocytes infected with adenovirus carrying sh-PRMT4 or sh-NC after treatment as described in part (B).

*P<0.05 indicates a significant difference between the Ad-PRMT4 and Ad-ctrl groups. #P<0.05 indicates a significant difference between the sh-PRMT4 and sh-NC groups. §P<0.05 indicates a significant difference between the insulin-treated and insulin+CL316,243-treated groups.
FFAs release in vitro. Lipolysis, a process in which triglycerides stored in adipose tissue are broken down and released into the bloodstream as glycerol and free fatty acids, is characterized by reduced mass of WAT, smaller adipocyte diameter and higher serum FFAs level.\(^{38-40}\) We found that PRMT4 overexpression induced fat pads loss in diabetic mice, which was contradictory to the consequence of its promotion of lipid synthesis. The reason for explaining this phenotype might be that the effect of PRMT4 on activating lipolysis overcomes the effect on promoting lipid synthesis under some conditions, such as type 1 diabetic mellitus and fasting.

As the cause of the higher levels of serum triglycerides and FFAs in the diabetic mice, the PRMT4 effect of promoting lipolysis is consistent with its effect of promoting lipid synthesis. To further study the effect of PRMT4 on insulin deficiency-induced lipolysis, we also explored the function of PRMT4 in the fasted mice, in which the activation of lipolysis was caused by insulin deficiency.\(^{41}\) In this model, more fat pads loss and higher serum FFAs levels were also observed in PRMT4-overexpressed mice. We also ascertained that insulin deficiency-associated lipolysis might depend on PRMT4 in adipocytes. Based on all these results we inferred that PRMT4 might promote lipolysis induced by insulin deficiency.

Lipolysis depends on the activity of three enzymes: HSL, ATGL, MAGL, each of which possesses a distinct regulatory mechanism.\(^{42}\) In our experiments, we found that PRMT4 upregulated the expression levels of ATGL, MAGL, HSL and activated HSL phosphorylation in T1DM mice and fasted mice. Meanwhile, HSL phosphorylation was activated by PRMT4 in catecholamines stimulated adipocytes. We speculated that PRMT4 promoted lipolysis by up-regulating the expression of these enzymes and activating their activity. But how PRMT4 regulates the transcription of genes related to lipolysis remains unclear. Previous studies had demonstrated that PRMT4 induce autophagy, lysosomes and lipid metabolism in mouse embryonic fibroblasts (MEFs).\(^{43}\) Moreover, overactivation of the autophagic digestion of lipid droplets resulted in increased levels of fatty acids. The previous study had also demonstrated that HSL/ATGL-independent lipolysis relies on the form of autophagy.\(^{44}\) On the bases of these studies, it is suggested that PRMT4 may promote lipid droplet breakdown and the release of FFAs by activating autophagy. However, these possibilities require further research for their confirmation.

Since PRMT4 activates lipolysis in diabetic mice, its inhibitors can be applied to inhibit lipolysis. Selective inhibitors of PRMT4 have been generated and confirmed to have antitumor effects.\(^{45,46}\) The effects of PRMT4 inhibitors on hypertriglyceridemia need further study. We believe that such inhibitors may be a potential therapeutic strategy for clinical dyslipidemia.

This study had the following limitations: (i) Only the gain-of-function effect was determined, whereas the loss-of-function effect on the metabolic phenotype was not evaluated. In our future study, adipocyte-specific PRMT4-knockout mice and PRMT4 inhibitors will be applied to explore the role of PRMT4 in mice with T1DM. (ii) Whether the specific mechanism of how PRMT4 affects lipolysis be related to autophagy has not been confirmed by experiments. Subsequent studies will systematically explore the specific molecular mechanisms underlying the effect of PRMT4 on lipolysis.

In conclusion, our results suggested that PRMT4 promotes lipolysis, increases the serum FFA content, results in hypertriglyceridemia regulated by lipolysis-related gene expression in T1DM mice. PRMT4 may be a new therapeutic target for ameliorating dyslipidemia of T1DM patients.

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Disclosure

The authors report no conflicts of interest in this work.

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