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Heparan sulfate in pancreatic β-cells contributes to normal glucose homeostasis by regulating insulin secretion

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ABSTRACT

Heparan sulfate (HS), a linear polysaccharide, is involved in diverse biological functions of various tissues. HS is expressed in pancreatic β-cells and may be involved in β-cell functions. However, the importance of HS for β-cell function remains unknown. Here, we generated mice with β-cell-specific deletion of *Ext1* (βExt1CKO), which encodes an enzyme essential for HS synthesis, to investigate the detailed roles of HS in β-cell function. βExt1CKO mice decreased body weights compared with control mice, despite increased food intake. Additionally, βExt1CKO mice showed impaired glucose tolerance associated with decreased insulin secretion upon glucose challenge. Glucose-induced insulin secretion (GIIS) from isolated βExt1CKO islets was also significantly reduced, highlighting the contribution of HS to insulin secretion and glucose homeostasis. The gene expression essential for GIIS was decreased in βExt1CKO islets. *Pdx1* and *MafA* were downregulated in βExt1CKO islets, indicating that HS promoted β-cell development and maturation. BrdU- or Ki67-positive β-cells were reduced in βExt1CKO pancreatic sections, suggesting the involvement of HS in the proliferation of β-cells. Moreover, insufficient vascularization in βExt1CKO islets may contribute to central distribution of α-cells. These data demonstrate HS plays diverse roles in β-cells, and that loss of HS leads to insufficient insulin secretion and dysregulation of glucose homeostasis.

1. Introduction

Heparan sulfate (HS) is a highly sulfated linear polysaccharide abundantly distributed on the cell surface [1]. HS consists of repeated sequence of disaccharide. HS elongation proceeds by alternating addition of *N*-acetylglucosamine and glucuronic acid in the presence of exostosin1 (Ext1) and exostosin2 (Ext2) [2,3]. Ext2 acts as a molecular chaperone delivering Ext1 to the Golgi apparatus; Ext1 predominantly exerts catalytic activity to synthesize HS [4]. Synthesized HS chains are further modified by various enzymes, including sulfotransferases. They possess high negative charge density, thereby attracting positively charged molecules, such as growth factors and cytokines [5]. This interaction between HS and active molecules at the cell surface enhances ligand-receptor encounters, resulting in the augmentation of intracellular signaling. In particular, the essential role of HS in the binding of fibroblast growth factors (FGF) to FGF receptor (FGFR) is well documented [6].

Extensive investigations reveal the essential roles of HS in development, differentiation and maturation [7]. Although HS is present in pancreatic β-cells of mice and human [8], the importance of HS in β-cells remained unknown. Previously, we investigated the roles of Exostosin-like 3 (Extl3), another enzyme involved in HS synthesis, to elucidate the importance of HS in β-cell functions and showed that *Extl3* heterozygous deletion in mice did not affect β-cell function [9]. On the other hand, recent clinical studies have

Abbreviations: Pdx1, pancreatic and duodenal homeobox 1; Maf A, v-maf musculoaponeurotic fibrosarcoma oncogene homolog; BrdU, bromodeoxyuridine.

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shown that heterozygous loss-of-function mutations in *EXT1* or *EXT2* impair insulin secretion [10], indicating the stronger impact of EXT1 or EXT2 on the regulation of β-cells. Our previous study also phenotyped conditional *Extl3* knockout mice by using *Extl3* flox mice and *insulin2*-Cre transgenic mice. Recent studies showed that *insulin2* promoter was also active in hypothalamus [11] and hypothalamic neurons controlled glucose-induced insulin secretion [12]. Thus, we could not rule out the possibility that neuronal expression of Cre recombinase substantially affected the phenotypes of conditional *Extl3* knockout mice [9]. In the present study, we crossbred *Ext1* flox mice [13] with newly established *insulin1-Cre* transgenic mice which had no neuronal expression of Cre recombinase [14]. We generated β-cell-specific *Ext1*-deficient (*insulin1-Cre;Ext1*$^{flox/flox}$; called βExt1CKO hereafter) mice to properly evaluate the roles of HS in β-cells *in vivo*.

2. Materials and methods

2.1. Mice

Creation of the loxP-modified *Ext1* allele (Ext1 flox) and insulin1-Cre transgenic mice was previously described [13,14]. Labo MR Stock (2.31 kcal/g; NOSAN Yokohama, Japan) and Quick FAT (4.25 kcal/g; CLEA, Tokyo, Japan) were used as a normal diet and a high-fat diet, respectively. All experimental procedures used in this study were approved by the Principles for Care and Use of Research Animals of Tohoku University, Sendai, Japan. All experiments involving animals are reported *in vivo* experiments guidelines [15,16].

2.2. Quantitative RT-PCR

RNA isolation, reverse transcription, and PCR were performed as described previously [17]. The primers used in this study were shown in Supplementary Table 1.

2.3. Glucose and insulin tolerance tests

Glucose (Wako, Osaka Japan; 2 g/kg body weight) and insulin (Wako; 0.75 U/kg body weight) were injected intraperitoneally. Both tests were performed as described previously [18].

2.4. Measurement of HS, insulin, glucagon and adiponectin concentrations by ELISA

The concentrations of HS, insulin, glucagon, and adiponectin were determined by ELISA. The ELISA kits used in this study are shown in Supplementary Table 2. For glucagon measurement, mice were fasted for 24 h before the experiments.

2.5. Determination of insulin and DNA content in pancreatic islets

Pancreatic islets were isolated by collagenase digestion method [19]. The pancreatic islet insulin and DNA contents were then measured as described previously [20].

2.6. Measurement of insulin secretion from isolated pancreatic islets

Batches of 15 pancreatic islets were pre-incubated at 37 °C for 30 min in Krebs-Ringer bicarbonate buffer containing 2.8 mM glucose at 37 °C with 95% O_2 and 5% CO_2. After pre-incubation, insulin secretion was measured as described previously [21].

2.7. Immunohistochemical analysis

After embedding in paraffin, tissues were sectioned and stained. The antibodies used in this study are shown in Supplementary Table 3. For BrdU immunostaining, BrdU solution (100 mg/kg body weight; Roche, Basel, Switzerland) was intraperitoneally injected, and pancreatic tissues were removed the following day. Fluorescent images were captured using a Nikon C2si microscope (Nikon, Tokyo, Japan). A BZ-9000 Fluorescence Microscope with the Hybrid Cell Count image analysis program (Keyence, Osaka, Japan) were used for morphometric analysis.

2.8. Western blotting

Islets isolated from five mice were used for western blotting. The 1st antibodies were incubated at 4 °C overnight and the 2nd antibodies were incubated at room temperature for 1 h. The antibodies used in this study were shown in Supplementary Table 3.

2.9. Statistical analysis

Experiments were analyzed using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA) for statistical analysis. All *p* values were calculated using Student's *t* tests with unpaired samples. Data are presented as means ± SEMs.

3. Results

3.1. HS in βExt1CKO islets was significantly lower than that in control islets

Ext1 flox mice (*Ext1*$^{flox/flox}$; called control) were used as control animals for this study. In βExt1CKO islets, Cre-mediated DNA recombination of the *Ext1* gene was confirmed by genomic PCR (Supplementary Fig. 1A). Quantitative RT-PCR analysis showed that *Ext1* mRNA expression was decreased in βExt1CKO islets without compensatory upregulation of other genes involved in HS synthesis (Supplementary Fig. 1B). The amount of HS in βExt1CKO islets was decreased by about 40% (Supplementary Fig. 1C).

3.2. βExt1CKO mice had impaired glucose tolerance with insufficient insulin secretion

The body weights of βExt1CKO mice were significantly reduced compared with those of control mice fed a normal diet (ND) or a high-fat diet (HFD) (Fig. 1A and Supplementary Fig. 2A). However, the amount of food intake in βExt1CKO mice was increased (Fig. 1B and Supplementary Fig. 2B). Adiponectin, which increases food intake and decreases body weights [22], was significantly increased in the plasma of βExt1CKO mice (Fig. 1C). Although the changes of blood glucose levels after glucose challenge were not observed in young βExt1CKO mice (8–12 weeks old) fed ND, aged βExt1CKO mice (more than 12 months of age) showed impaired glucose tolerance (IGT) (Supplementary Figs. 2C and D), suggesting that *Ext1* deficiency gradually impaired β-cell functions after ND consumption. Moreover, young βExt1CKO mice fed HFD showed significantly higher blood glucose levels after glucose loading than control mice fed HFD (Fig. 1D). Plasma insulin levels after glucose challenge were significantly lower in βExt1CKO mice fed HFD (Fig. 1E), although insulin sensitivity of βExt1CKO mice was not changed compared with that of control mice during insulin tolerance tests (Fig. 1F). These results demonstrated that β-cell dysfunction in βExt1CKO mice caused insufficient insulin secretion coupled with high blood glucose levels after glucose challenge. Subsequent experiments were performed using 8–12 weeks old

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