A high-fat/high-sucrose diet induces WNT4 expression in mouse pancreatic β-cells

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Abstract

Aims/Introduction: Several lines of evidence suggest that dysregulation of the WNT signaling pathway is involved in the pathogenesis of type 2 diabetes. This study was performed to elucidate the effects of a high fat/high sucrose (HF/HS) diet on pancreatic islet functions in relation to modulation of WNT ligand expression in β-cells.

Materials and Methods: Mice were fed either standard mouse chow or a HF/HS diet from 8 weeks of age. At 20 weeks of age, intraperitoneal glucose tolerance tests were performed in both groups of mice, followed by euthanasia and isolation of pancreatic islets. WNT-related gene expression in islets and MIN6 cells was measured by quantitative real-time RT-PCR. To explore the direct effects of WNT signals on pancreatic β-cells, MIN6 cells were exposed to recombinant mouse WNT4 protein (rmWNT4) for 48 h, and glucose-induced insulin secretion were measured. Furthermore, *Wnt4* siRNAs were transfected into MIN6 cells, and cell viability and insulin secretion were measured in control and *Wnt4* siRNA-transfected MIN6 cells.

Results: Mice fed the HF/HS diet were heavier and their plasma glucose and insulin levels were higher compared with mice fed standard chow. *Wnt4*, *Wnt5b*, *Ror1*, and *Ror2* expression was upregulated, while *Fzd4*, *Fzd5*, *Fzd6*, *Lrp5*, and *Lrp6* expression was downregulated in islets of mice fed the HF/HS diet. *Wnt4* was the most abundantly

expressed WNT ligand in β-cells, and its expression was increased by the HF/HS diet. Although exposure to recombinant mouse WNT4 protein for 48 h did not alter glucose-induced insulin secretion, it was significantly reduced by knockdown of *Wnt4* in MIN6 cells.

Conclusions: We demonstrated that the HF/HS diet-induced increase of WNT4 signaling in β-cells is involved in augmentation of glucose-induced insulin secretion and impaired β-cell proliferation. These results strongly indicate an essential role of WNT4 in the regulation of β-cell functions in mouse pancreatic islets.

Keywords:

WNT4, high fat/high sucrose diet, pancreatic islet, β-cell, insulin secretion

Running title:

Role of WNT4 in β-cell functions

1. Introduction

Surplus intake of dietary fat and sugar is extremely relevant to the pathogenesis of type 2 diabetes. Mice fed a high fat/high sucrose (HF/HS) diet develop insulin resistance and hyperinsulinemia, and exhibit hyperplasia of pancreatic β-cells (1). The susceptibility and phenotype vary depending on the genetic background, and it is feasible that accumulation of visceral fat contributes to the development of insulin resistance. However, the precise mechanisms by which excessive intake of a HF/HS diet causes β-cell hyperplasia and hyperinsulinemia remain to be elucidated.

WNT signaling pathways play critical roles in the differentiation, proliferation, death, and function of various cell types (2,3,4). Aberrant WNT signaling is associated with various diseases including cancer (5, 6). Dysregulation of the WNT signaling pathway is also involved in the pathogenesis of type 2 diabetes in relation to expression of *TCF7L2*, the most responsible gene for type 2 diabetes, which encodes a key component of the WNT signaling pathway (7).

The *Wnt* gene was first identified in 1982 and is named the *Int* gene in mice (8). Subsequently, the Drosophila *int1* homolog was found to be wingless. As a result, both genes were recognized as the *Wnt* gene (9). WNTs are a large family of 19 secreted carbohydrate- and lipid-modified proteins including Wnt1, Wnt2, Wnt2b (Wnt13), Wnt3,

Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a (Wnt14),

Wnt9b (Wnt14b), Wnt10a, Wnt10b, Wnt11, and Wnt16 (10).

The most studied WNT ligand is WNT 3a. The addition of purified WNT3a protein

to cultured β-cells or islets promotes expression of *Pitx2*, a direct target of WNT signaling,

as well as Cyclin D2, an essential regulator of the β-cell cycle and proliferation (11).

WNT4 completely blocks WNT3a-stimulated β-cell growth and insulin secretion (12). In

the present study, we examined the putative relevance of WNT4 signaling to the

pathogenesis of diabetes and insulin resistance under a HF/HS condition in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from Nippon CLEA (Shizuoka, Japan). They

were housed in standard cages with free access to water under a 12 h light/12 h dark cycle

from 8 to 20 weeks of age, and fed either control chow (347 kcal/100 g, 12% fat and 28%

protein; Nippon CLEA) or a HF/HS diet (592 kcal/100 g, 70% fat, 14% sucrose, 3% other

carbohydrates, and 13% protein; Oriental Yeast, Tokyo, Japan). All mice were treated

according to the guidelines for the care and use of laboratory animals of Kurume

University School of Medicine based on the National Institutes of Health Guidelines. All

efforts were made to ensure minimal suffering.

2.2. Experimental protocols for mice fed control chow or the HF/HS diet

At 20 weeks of age, an intraperitoneal glucose tolerance test was performed by injection of glucose (1 g/kg body weight dissolved at 10 v/v% in distilled water) intraperitoneally after overnight fasting. Blood was obtained from the tail vein, and glucose concentrations were measured by the glucose dehydrogenase method using Free Style (Nipro, Osaka, Japan) at 0, 30, 60, and 120 min after glucose injection. Then, all mice were euthanized under anesthesia for the following experiments. Blood samples were collected from the inferior vena cava and centrifuged (3000 g, 10 min), and sera were collected and stored at -80°C until assaying the insulin concentrations. Pancreatic islets were isolated from the harvested pancreas by collagenase digestion as described previously (13).

The pancreas was fixed in 4% paraformaldehyde and embedded in paraffin. Sections of the pancreas were incubated with a rabbit polyclonal antibody against WNT4 (ab91226; Abcam, Cambridge, UK) at a dilution of 1:20 for 24 h. The sections were then washed and incubated with fluorochrome-conjugated goat anti-rabbit IgG H&L (ab150078; Abcam) for 1 h at room temperature. Immunostaining of insulin, glucagon, and somatostatin was performed using a guinea pig polyclonal anti-insulin antibody

(ab7842; Abcam), goat polyclonal anti-glucagon (N-17) antibody (sc-7780; Santa Cruz

Biotechnology, Santa Cruz, CA, USA), and goat polyclonal anti-somatostatin antibody

(sc-7819; Santa Cruz Biotechnology), respectively, followed by corresponding

fluorochrome-conjugated secondary antibodies (Abcam).

2.3. Protocols for the culture and incubation experiment using MIN-6 cells

The MIN6 pancreatic β-cell line, which was kindly provided by Prof. Jun-ichi

Miyazaki (Osaka University) (14), was maintained in Dulbecco's modified Eagle's

medium (DMEM) (25 mmol/L glucose) containing 10% (v/v) fetal bovine serum, 50

μmol/L β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C with

5% CO_2. Twenty-four hours before transfection, the cells were trypsinized and cell

numbers were counted. Then, they were transferred and placed in culture medium. *Wnt4*

siRNAs (Thermo Scientific, Lafayette, CO, USA) and a control siRNA were transfected

in accordance with the manufacturer's instructions at a working concentration of 50

nmol/L using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA). Transfected

cells were cultured for 48 h until subsequent experiments.

In another series of experiments, recombinant mouse WNT4 (rmWNT4; R&D

Systems, Abingdon, UK) was added to the incubation medium at concentrations of 0.2 or

0.5 μg/ml. The cells were then cultured for 48 h until subsequent experiments.

On the day of the experiment to measure insulin secretion, MIN6 cells were harvested and seeded in 24-well plates at a density of 1.5×10^5 cells per well. The cells were cultured for 72 h and pre-incubated at 37°C for 60 min in Hepes-Krebs buffer (118.4 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 2.4 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 20 mmol/L $NaHCO_3$, 2.2 mmol/L glucose, and 10 mmol/L Hepes) containing 0.5% (w/v) bovine serum albumin. Then, Hepes-Krebs buffer was changed into the one containing 22 mmol/L glucose, and incubated for the next 60 min in . Culture media containing either 2.2 or 22 mmol/L glucose were collected and stored at -80°C until assaying the insulin concentrations.

A stock solution was prepared by dissolving palmitate (R&D Systems Inc.) in 95% ethanol to 25 mmol/L. This solution was diluted in glucose-free DMEM containing 2% fatty acid-free bovine serum albumin (Gibco, Grand Island, NY, USA) to 2.5 mmol/L and then mixed at 37°C for 1 h. The final concentration of palmitate in the medium was adjusted to 0.4 mmol/L. After 24 h of exposure to palmitate, cell lysates were subjected to experiments for measurements of *Wnt4* and *Chop* gene expression.

2.4. Measurements of serum insulin concentration, gene expression and cell proliferatoin

Insulin concentrations in serum were measured by an ultrasensitive mouse insulin

ELISA kit (Shibayagi, Gunma, Japan). Insulin resistance was evaluated by homeostatic model assessment-insulin resistance (HOMA-IR) [fasting blood glucose (mg/dL) × fasting blood insulin (μU/mL) / 405].

Total RNA in pancreatic islets was extracted with RNA-Bee (Cosmo Bio, Tokyo, Japan), and that in MIN-6 cells was extracted with an RNeasy Micro kit (Qiagen, CA). A PrimeScript RT Reagent Kit with gDNA Eraser was employed to reverse transcribe the total RNA to cDNA (PR047A; Takara, Kusatsu, Japan). Subsequently, quantitative real-time RT-PCR was performed with SYBR Premix Ex Taq II (RR420A; Takara) using a StepOnePlus (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. The expression of individual genes was normalized to either *Gapdh* expression for pancreatic islets or *Tbp expression* for MIN-6 cells.

Cell proliferation was measured by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. Absorbance at 450 nm was determined by a microplate reader (Bio Rad, Hercules, CA, USA). Cell viability was calculated as follows: viable cell number (%) = (mean absorbency in test wells) / (mean absorbency in control wells) × 100.

2.5. Statistical analysis

All statistical analyses were performed using SPSS Statistics Ver. 23 (IBM Corp. NY). Statistical significance was determined by the Mann-Whitney U-test. Data are presented as means ± standard deviation (S.D.). A p-value of less than 0.05 was considered to be statistically significant.

3. Results

Mice fed the HF/HS diet were heavier and exhibited higher fasting and post-load glucose levels at 20 weeks of age compared with mice maintained on standard chow (Fig. 1A, B). Additionally, fasting plasma insulin levels were significantly more elevated in mice fed the HF/HS diet than in those fed standard chow (Fig. 1C). HOMA-IR in mice fed the HF/HS diet was significantly higher than that in those fed standard chow (Fig. 1D). Insulin immunostaining revealed hypertrophic islets in the pancreas of 20-week-old mice fed the HF/HS diet (Fig. 1E, F). However, little difference was seen in the number and distribution of glucagon-positive α-cells in the islets regardless of diet (Fig. 1G, H). The expression of insulin genes in isolated islets was assessed by quantitative real-time RT-PCR at 20 weeks of age. However, no significant difference was observed in the relative expression levels of *Ins1* or *Ins2* (Fig. 1I) between mice fed the HF/HS diet or standard chow.

We next analyzed the expression of WNT pathway-related genes in isolated islets (Fig. 2). Seven of the 19 WNT ligand family member genes were expressed at detectable levels in islets obtained from 20-week-old mice (Fig. 2A). As summarized in Table 1, the lowest cycle threshold value was obtained for *Wnt4*. *Wnt4* and *Wnt5b* expression was upregulated in islets of mice fed the HF/HS diet compared with standard chow-fed mice (Fig. 2A). In contrast, HF/HS diet-fed mice displayed diminished *Wnt2b*, *Wnt11*, and *Wnt14* expression in islets (Fig. 2A). We analyzed the expression of genes associated with WNT signaling, revealing that nine of the 10 known Fzd receptor genes were expressed in the islets. HF/HS diet-fed mice showed notably lower *Fzd4*, *Fzd5*, and *Fzd6* expression levels compared with mice fed standard chow (Fig. 2B). Expression of the two co-receptors, *Lrp5* and *Lrp6*, was also decreased in islets obtained from HF/HS diet-fed mice (Fig. 2C). In contrast, significant increases of *Ror1* and *Ror2* were observed in islets of HF/HS diet-fed mice (Fig. 2D).

Immunostaining of WNT4 and insulin in serial pancreas sections from 20-week-old mice fed standard chow revealed that non-β-cells located at the periphery of islets highly produced WNT4 (Fig. 2E–G). Interestingly, compared with islets from mice fed standard chow, WNT4 staining was much stronger in mice fed the HF/HS diet, especially in the central area of islets (Fig. 2H, I). These results suggested that the HF/HS diet increased

Wnt4 expression in β-cells, prompting us to assess a possible direct effect of fatty acids on *Wnt4* gene expression. However, as shown in Fig. 2J, treatment with palmitate led to a significant reduction of *Wnt4* expression in MIN6 cells, suggesting that other factor(s) related to insulin resistance may be involved in the upregulation of WNT4 under a HF/HS diet *in vivo*. Reciprocal induction of the transcription factor *CHOP* by the saturated fatty acid indicated aggravation of endoplasmic reticulum (ER) stress due to lipotoxicity in MIN6 cells.

To identify cells that were strongly positive for WNT4, we stained three serial sections with anti-glucagon, anti-WNT4, and anti-somatostatin antibodies, respectively (Fig. 3A–C). Most cells showing strong positivity for WNT4 were glucagon-containing cells. In contrast, somatostatin-containing cells did not show strong staining for WNT4 (Fig. 3D, E).

Exposure of MIN6 cells to rmWNT4 protein for 48 h did not affect glucose-induced insulin secretion (Fig. 4A). The reduction of *Wnt4* expression by transfecting MIN6 cells with *Wnt4*-specific siRNAs was confirmed by quantitative RT-PCR (Fig. 4B). The numbers of viable cells were unchanged by knockdown of *Wnt4* expression (Fig. 4C). Although there was no effect on the expression of insulin genes (Fig. 4D, E), glucose-induced secretion of insulin was significantly reduced by *Wnt4* knockdown in MIN6 cells

(Fig. 4F).

4. Discussion

Various lines of evidence indicate that β-catenin/TCF7L2-dependent WNT signaling plays crucial roles in pancreas development, islet cell proliferation, and insulin secretion. Cre-mediated β-cell-specific deletion of β-catenin inhibits insulin secretion and glucose intolerance in mice. It was suggested that the lack of β-catenin might delay β-cell maturation, resulting in abnormal glycemia and insulinemia owing to β-cell dysfunction (15). Moreover, the reduction of β-catenin in β-cell lines using siRNA or pyrvinium, an inhibitor of the β-catenin signaling pathway, attenuates glucose- and KCl-stimulated insulin secretion (16).

Depending on their functions, WNT ligands are classified into two categories. WNT1, WNT2, WNT2b, WNT3, WNT3a, WNT7a, WNT8, WNT8b, and WNT10a belong to the canonical signaling pathway, while WNT4, WNT5a, and WNT11 belong to the non-canonical signaling pathway (17). Former studies of WNT ligands have focused on the role of WNT3a that activates the canonical β-catenin signaling in β-cells. WNT4 as a non-canonical ligand is known to be involved in kidney and gonadal gland development (18, 19). It has been recently reported that expression of *Wnt4* is abundant in pancreatic islets

and upregulated in insulin-resistant model mice (20). However, the precise role of Wnt4

in diabetes and insulin resistance has not been elucidated so far. In pancreatic islets and

INS-1 cells, WNT4 inhibits WNT3a-mediated increases in proliferation and glucose-

stimulated insulin secretion (12). The abundance of WNT4 in pancreatic islets and its

increase in response to insulin resistance implies potential roles of WNT4 in pancreatic

islets independent of WNT3a.

Mice fed the HF/HS diet exhibited obesity, insulin resistance, and glucose intolerance.

HF/HS diet-fed mice had larger pancreatic islets that predominantly consisted of β-cells.

In line with former reports (21, 22), *Wnt4* was the most abundantly expressed WNT-

related gene in the islets. Consistent with a study by Bowen et al. [9], immunostaining

revealed that the protein level of WNT4 was greater in α-cells than in β-cells in the

pancreas of standard chow-fed mice. We also found that the HF/HS diet increased *WNT4*

expression in β-cells to a comparable level as that in α-cells. These observations suggest

that WNT4 plays an essential role in β-cell proliferation or alterations of the insulin

secretory response induced by the HF/HS diet.

Next, we assessed the effect of *Wnt4* knockdown on MIN6 cell proliferation using

specific siRNAs. *Wnt4* knockdown resulted in no significant difference in the number of

viable cells, suggesting that WNT4 does not affect the regulation of MIN6 cell

proliferation. In rat INS-1 cells, the role of endogenous WNT4 in cell proliferation has been controversial (12, 23). Overall, the role of endogenously expressed WNT4 may vary depending on the cell type.

Wnt4 knockdown did not affect *Ins1* or *Ins2* expression. In contrast, glucose-induced insulin secretion was significantly suppressed in MIN6 cells treated with *Wnt4*-specific siRNAs, suggesting that WNT4 may be involved in glucose-induced vesicle transport of insulin in MIN6 cells. Therefore, upregulation of *Wnt4* expression may be associated with compensatory enhancement of the insulin secretory response, but not insulin biosynthesis, in diet-induced insulin-resistant mice. In line with former reports (12, 23), the addition of exogenous WNT4 did not exert a notable effect on insulin secretion. Although its precise mechanisms remain unknown, it is feasible that WNT4 is secreted from β-cells and acts as a ligand for its receptor in an autocrine/paracrine manner, thereby prohibiting additional ligand binding.

WNT4 dominantly produced in β-cells under the HF-HS condition might play a role in glucose-induced insulin secretion. Non-canonical WNT signals include calcium release from the ER and the resultant elevation of intracellular calcium, which may augment insulin release from β-cells. However, to explicate the precise mechanism of the WNT4 action in β-cells, further studies are required. The reduced expression of *Lrp5* and *Lrp6*,

which are co-receptors for transduction of β-catenin-dependent signals, and increased expression of *Ror1* and *Ror2*, which are co-receptors involved in non-β-catenin-dependent WNT signaling, likely shift the balance between canonical and non-canonical signals toward the latter. The altered expression of WNT co-receptors may be involved in the diabetogenic effect of the HF/HS diet because LRP5-deficient mice also exhibit impaired glucose tolerance (24).

5. Conclusion

This study demonstrated that long term feeding with a HF/HS diet profoundly altered the expression of WNT signaling genes in pancreatic islets, and that expression of *Wnt4* was significantly increased in β-cells. WNT4 signaling plays a pivotal role in regulating glucose-induced insulin secretion. Comprehensive understanding of the WNT4 signaling pathway may facilitate development of new therapeutics for type 2 diabetes.

Disclosure

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Figure legends

Figure 1. Body weight (A), fasting and post-load plasma glucose concentrations (B), fasting plasma insulin levels (C), and HOMA-IR (D) of 20-week-old mice fed standard

chow (open column, circle) or the high fat/high sucrose (HF/HS) diet (closed column, square). Mean ± S.D., *p<0.05, **p<0.01, n=6. Immunostaining of insulin (E, F) and glucagon (G, H) in islets of 20-week-old mice fed standard chow (E, G) or the HF/HS diet (F, H). Scale bars indicate 100 μm in E and F, and 50 μm in G and H. Relative expression of *Ins1* and *Ins2* (I) in islets of 20-week-old mice fed standard chow (open column) or the HF/HS diet (closed column). Mean ± S.D., n=6.

Figure 2. Relative expression of WNT ligands (A), Frizzled receptors (B), non-Frizzled WNT receptors (C), and co-receptors (D) in the islets of mice fed the HF/HS diet. Data are shown as ratios to those in mice fed standard chow. Means ± S.D., *p<0.05, **p<0.01 vs. the values of mice fed standard chow. Immunostaining of WNT4 (E) and insulin (F), and a merged image (G) in an islet of a mouse fed standard chow. Immunostaining of WNT4 in islets of mice fed standard chow (H) or the HF/HS diet (I). Scale bars indicate 50 μm. Relative expression of *Wnt4* and *Chop* in MIN6 cells treated with or without 0.4 mM palmitate for 24 h. Means ± S.D., *p<0.05, **p<0.01 (J).

Figure 3. Immunostaining of WNT4 (A), glucagon (B), and somatostatin (D) in serial sections of an islet of a 20-week-old mouse fed standard chow. Merged image of Wnt4

and glucagon staining (C). Merged image of Wnt4 and somatostatin staining (E).

GCG; glucagon, SST; somatostatin.

Figure 4. Effect of recombinant mouse WNT4 on glucose-induced insulin secretion in

MIN6 cells (A), n=4. Knockdown of *Wnt4* expression by specific siRNAs (B), n=4. Effect

of *Wnt4* knockdown on the number of viable MIN6 cells (C), n=3, expression of *Ins1* (D)

and *Ins2* (E), and glucose-induced insulin secretion (F), n=4. Means ± S.D. *p<0.05,

**p<0.01 vs. control siRNA.

Table 1. Cycle threshold (CT) values in real-time RT-PCR of WNT ligand, receptor, and

co-receptor genes.