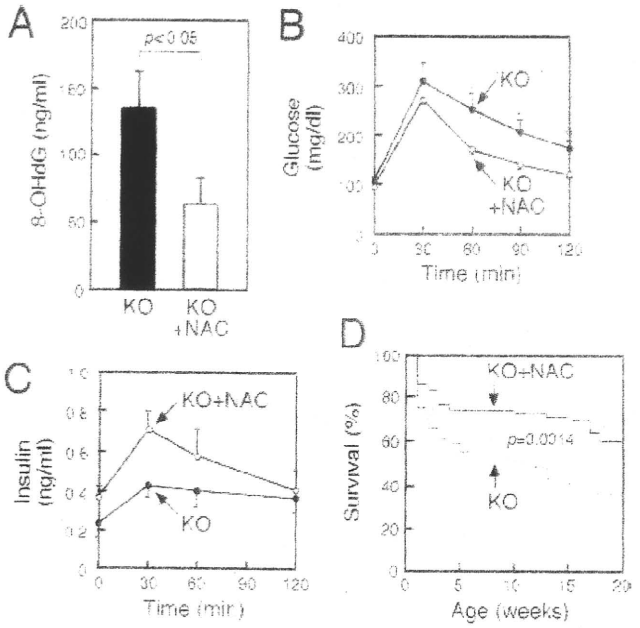

FIGURE 6. **NAC treatment improves deficiencies of glucose uptake and insulin secretion seen in SMS1-KO mice.** SMS1-KO mice supplied with normal (*KO*) or NAC-containing drinking water (*KO+NAC*) for 20–24 weeks were used for the following analysis. *A*, concentration of urinary 8-OHdG, a marker of ROS generation, was assessed (KO, $n = 8$; KO+NAC, $n = 10$). *B*, blood glucose levels based on glucose tolerance tests. Mice were deprived of food for 16 h and injected with 1 mg/kg glucose. Blood was withdrawn at times indicated. Blood glucose was measured using the glucose oxidase method (*KO*, $n = 7$; *KO+NAC*, $n = 7$). *C*, serum insulin levels derived from glucose tolerance tests. The amounts of insulin in the serum obtained from glucose tolerance tests performed in *B* were measured by an ELISA (*KO*, $n = 7$; *KO+NAC*, $n = 7$). *D*, survival curve of SMS1-KO supplied with normal (*KO*, $n = 87$) or NAC-containing drinking water (*KO+NAC*, $n = 72$). *, $p < 0.05$; **, $p < 0.01$.

DISCUSSION

Although investigators have examined SMS1 function in cell growth and apoptosis in cell culture, the significance of SMS1 loss *in vivo* has not been reported. Here, we generated SMS1-KO mice and found that SMS1-KO mice exhibited moderate neonatal lethality, reduced body weight, and loss of fat tissues mass, suggesting that they might have metabolic deficiency. We further found that they were hyperglycemic and showed insulin secretion deficiencies. The deficiencies were not attributable to death of pancreatic β-cells. Isolated SMS1-KO islets exhibited severely impaired ability to release insulin, dependent on glucose stimuli, indicating that impaired insulin secretion from pancreatic β-cells is a primary cause of insulin secretion deficiency observed in SMS1-KO mice. Therefore, in this study, we focused on analyzing how SMS1 deletion disturbs insulin secretion from pancreatic β-cells.

Lipid composition analysis revealed that the amount of sphingomyelin species was reduced whereas that of ceramide species was increased in SMS1-KO islet. These results appeared to be reasonable, because ceramide and sphingomyelin are enzymatic substrate and product of SMS reaction, respectively. However, reduction of the amounts of sphingomyelin in SMS1-KO islet appeared to be small. Although the reason is unclear, we consider that other sphingomyelin synthetic or influx pathway is present. For example, a portion of SMS2 may be reside in Golgi complex, and partially participate in sphingomelin synthesis in Golgi complex to compensate for SMS1 ablation. Alternatively, it is also possible that sphingomyelin, which is derived from food intake, flow into the cell by endocytosis.

Analysis of lipid content in pancreatic mitochondria indicated that the amount of sphingomyelin species was reduced in SMS1-KO mice, whereas that of ceramide species was increased. Indeed, intracellular levels of ceramide in ER and mitochondria are reportedly increased when the ceramide transfer protein, CERT, is ablated (17). These authors also found that mitochondrial ceramide accumulation is associated with mitochondrial degeneration, including the capacity to generate ATP. These conclusions are similar to our observations that SMS1-KO islets exhibited deficiencies in ATP production following glucose stimuli. In addition, mitochondria of SMS1-KO islet showed other anomalies, such as hyperpolarized membrane potential and increased ROS production. These results are consistent with previous reports indicating that increased ROS generation induced by knockdown of the mitochondrial ROS detoxification enzyme, nicotinamide nucleotide transhydrogenase (Nnt), uncouples mitochondrial metabolism of pancreatic β-cells, leading to impaired ATP production and insulin secretion deficiency (41, 42). In addition, we observed higher expression of genes encoding mitochondrial respiratory chain complex components and transcription factors related to mitochondrial biogenesis. Up-regulation of these genes suggests that functional proteins are newly synthesized to compensate for ROS damage. In particular, mitochondrial complex I was highly expressed in SMS1-KO islets, which may promote higher membrane potential and increased ROS generation, as reported (40, 43). UCP2 was also up-regulated in SMS1-KO islets, in agreement with reports suggesting that the ROS-damaged β-cells express UCP2 to antagonize ROS production in mitochondria (44, 45). Overall, we estimate that mitochondrial respiration complexes in β-cells are functionally damaged by increased ceramide species in SMS1-KO mice and that electron leakage generates ROS, further damaging the mitochondrial respiration machinery. We further estimate that mitochondrial respiration complex components are up-regulated to restore mitochondria damaged by ROS, and that UCP2 is up-regulated to reduce oxidative damage caused by ROS generation.

Finally, we found that the anti-oxidant NAC treatment improved glucose uptake and rescued insulin secretion deficiencies seen in SMS1-KO mice, supporting the idea that ROS production underlies insulin secretion deficiency seen in SMS1-KO pancreatic β-cells. These observations are noteworthy because these data suggest that increment of ceramide itself is not so much toxic, rather, the following ROS generation is very toxic. Altogether, our data suggest that SMS1 plays a critical role in regulating mitochondrial sphingolipid homeostasis and is required to control mitochondrial ATP and ROS production, which are important for insulin secretion in pancreatic β-cells.

It is explicit that SMS1 is important for whole body health, because SMS1-KO mice exhibit moderate neonatal lethality. And, SMS1 appears to be important for suppressing onset of ROS-related diseases, because NAC treatment prolonged the lifespan of SMS1-KO mice. These observations are consistent with previous reports that ROS functions in cell senescence (46, 47) and that reduction of ROS by NAC extends lifespan (48). Anyway, further experiments are necessary to reveal the complicated relationship between SMS1 function and ROS-related disease.

In this study, we demonstrates that manipulation of sphingolipid flux *in vivo* and consequent ceramide accumulation in pancreatic β-cells leads to defects in insulin secretion by oxidative stress probably imposed on mitochondria. Overall, our approach identifies an essential role for SMS1 in insulin secretion and provides molecular insight into the role of the *de novo* sphingolipid biosynthetic pathway in regulating the ROS generation pathway, which is related to metabolic disease.

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Similar incretin secretion in obese and non-obese Japanese subjects with type 2 diabetes

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ABSTRACT

Incretin secretion and effect on insulin secretion are not fully understood in patients with type 2 diabetes. We investigated incretin and insulin secretion after meal intake in obese and non-obese Japanese patients with type 2 diabetes compared to non-diabetic subjects. Nine patients with type 2 diabetes and 5 non-diabetic subjects were recruited for this study. Five diabetic patients were obese (BMI $\geqslant$ 25) and 4 patients were non-obese (BMI < 25). In response to a mixed meal test, the levels of immunoreactive insulin during 15–90 min and C-peptide during 0–180 min in non-obese patients were significantly lower than those in obese patients. Total GLP-1 and active GIP levels showed no significant difference between obese and non-obese patients throughout the meal tolerance test. In addition, there were no significant differences between diabetic patients and non-diabetic subjects. In conclusion, incretin secretion does not differ between Japanese obese and non-obese patients with type 2 diabetes and non-diabetic subjects.

Introduction

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are major incretins released from gut endocrine cells in response to nutrient ingestion. They have an important physiological role enhancing glucose-stimulated insulin secretion from the beta cells, the so-called incretin effect. In non-diabetic subjects, the quantitative contribution of this effect to the overall postprandial insulin secretion has been estimated to be 50–70% [1,2].

Type 2 diabetes is characterized by insulin resistance and relative insulin deficiency. In this type of diabetes, incretin effect is reported to be severely reduced [3]. GLP-1 retains insulinotropic activity, but its potency and rate of secretion is reduced. The secretion of GIP is usually preserved, but its effect on insulin secretion is lost or markedly reduced [4–8]. However, these findings were made in the Caucasian subjects with type 2 diabetes. In those studies, almost all subjects were obese or overweight and hyperinsulinemic. Their insulin secretion was preserved to some extent, but plasma glucose level increased due to severe insulin resistance. In contrast, in Japanese type 2 diabetic subjects, the mean body mass index (BMI) was 26.1, 23.5, 21.7 and 19.4 in men, 27.7, 24.1, 22.0 and 19.2 in women when divided into quartiles of BMI, indicating that we have type 2 diabetic patients both with normal weight and relatively high BMI (BMI $\geqslant$ 25) in Japan [9]. Indeed, in Japanese criteria, obesity is diagnosed when BMI is equal to or more than 25. Insulin secretion is usually preserved in obese, but not in non-obese subjects in Japanese type 2 diabetes. The clinical use of incretin-related drugs will soon explode in many developed countries, including Western and Asian countries. Therefore, it is quite important to know the baseline characteristics of incretin secretion in various diabetic patients, compared to non-diabetic subjects. In the present study, we investigated incretin and insulin secretion after meal intake in obese and non-obese Japanese patients with type 2 diabetes and non-diabetic subjects.

Materials and methods

Materials. Nine patients with type 2 diabetes and 5 non-diabetic subjects were recruited for this study. Five diabetic patients were obese (BMI $\geqslant$ 25), including 2 patients with oral hypoglycemic agents therapy and 3 patients with diet therapy alone. Four patients were non-obese (BMI < 25), including 3 patients receiving oral hypoglycemic agents and 1 patient with insulin therapy. The clinical characteristics of the patients are shown in Table 1. There was no significant difference in age, waist circumference (WC), visceral fat area (VFA), lipid profile and adiponectin level between any

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Table 1
Clinical characteristics of the study subjects.

	Obese diabetic patients ($n = 5$)	Non-obese diabetic patients ($n = 4$)	Non-diabetic subjects ($n = 5$)
Age (years)	59.2 ± 5.4	63.8 ± 8.2	51.0 ± 11.4
Duration (years)	4.4 ± 1.8	13.8 ± 9.3	–
Body mass index (kg/m^2)	27.7 ± 3.4[a]	21.8 ± 2.7[c]	23.8 ± 0.9
Waist (cm)	95.8 ± 6.8	83.2 ± 12.2	89.9 ± 4.2
Visceral fat area (cm^2)	183.4 ± 64.1	91.0 ± 54.9	117.2 ± 19.7
Hemoglobin A1c (%)	7.4 ± 1.8[b]	9.5 ± 1.5[b]	5.1 ± 0.3
SBP (mmHg)	136. ± 27.8	103.5 ± 6.6[b]	130.4 ± 9.8
DBP (mmHg)	85.2 ± 18.0	60.5 ± 3.0[b]	77.6 ± 6.2
LDL-C (mg/dl)	114.9 ± 39.5	91.5 ± 18.0	106.5 ± 58.0
HDL-C (mg/dl)	40.0 ± 6.2	52.0 ± 7.0	46.0 ± 11.3
TG (mg/dl)	149.4 ± 82.4	81.3 ± 46.2	142.4 ± 76.1
Adiponectin (μg/ml)	6.6 ± 2.5	8.2 ± 4.6	5.0 ± 2.2

[a] $P < 0.01$ vs non-diabetic subjects.
[b] $P < 0.05$ vs non-diabetic subjects.
[c] $P < 0.05$ vs obese diabetic patients.

two groups. There was also no significant difference in duration of diabetes between obese and non-obese patients. BMI in obese patients was significantly higher than that in non-obese patients (27.7 ± 3.4 vs 21.8 ± 2.7, $P < 0.01$). Hemoglobin A1c (A1C) in obese and non-obese diabetic patients was significantly higher than that in non-diabetic subjects (7.4 ± 1.8 vs 5.1 ± 0.3%, $P < 0.05$, obese diabetic patients vs non-diabetic subjects) (9.5 ± 1.5 vs 5.1 ± 0.3%, $P < 0.05$, non-obese diabetic patients vs non-diabetic subjects). Written informed consent was obtained from all participants with the approval of the ethics committee of Osaka University.

Mixed meal tolerance test. After hospitalization and discontinuation of all oral hypoglycemic agents, all diabetic patients were treated by insulin (or diet alone) at least for 2 weeks until fasting plasma glucose level became below 126 mg/dl. Mixed meal tolerance test was performed after an overnight fast. The test meal is authorized by the Japan Diabetes Society and the total caloric content of the test meal was 460 kcal (carbohydrates 56.5 g, protein 18 g, fat 18 g) (JANEF E460F18: Q.P. Corporation, Tokyo, Japan). The test meal was ingested within 15 min. Blood samples were collected at 0, 15, 30, 60, 90, 120, 150 and 180 min after the meal.

The levels of glucose, immunoreactive insulin (IRI), C-peptide and incretins (total GLP-1 and active GIP) in patients' and non-diabetic subjects' sera or plasma were measured at each time. Plasma glucose concentrations were measured by the glucose oxidase method. IRI and C-peptide levels were measured with enzyme immunoassay kits. To measure incretin levels, EDTA-coated tubes (1.5 μg/ml blood) containing an inhibitor of dipeptidyl peptidase (DPP)-4 (10 μl/ml blood; Linco Research Inc., MO, USA) were used. Total GLP-1 concentrations were measured using radioimmunoassay, using antiserum No. 89390 which is specific for the COOH-terminal end of the GLP-1 molecule and reacts equally with intact GLP-1 and the primary (NH2-terminally truncated) metabolite as described previously [10,11]. Active GIP concentrations were measured as described previously [12].

Statistical analysis. Data are presented as means ± standard deviation (SD). All statistical calculations were carried out using repeated-measures ANOVA using Statview (SAS Institute, Cary, NC). Values at single time points and areas under the curve (AUCs) were also compared by Mann–Whitney Test. P value < 0.05 was taken to indicate significant difference.

Results

Glucose concentrations in obese and non-obese diabetic patients were significantly higher than those in non-diabetic subjects. Glucose concentrations in non-obese patients tended to be higher than those in obese patients, but showed no significant difference (Fig. 1A). The levels of IRI and C-peptide showed no significant difference between obese patients and non-diabetic subjects. On the other hand, the levels of IRI during 15–60 min and C-peptide during 0–150 min in non-obese patients were significantly lower than those in non-diabetic subjects. The levels of IRI during 15–90 min and C-peptide during 0–180 min in non-obese patients were also significantly lower than those in obese patients (Fig. 1B and C). A fasting C-peptide-to-glucose ratio was 0.018 ± 0.006 ng dl/ml mg in obese patients, 0.005 ± 0.002 ng dl/ml mg in non-obese patients and 0.018 ± 0.008 ng dl/ml mg in non-diabetic subjects, respectively. The ratio in non-obese patients was significantly lower than that in obese patients and in non-diabetic subjects.

The levels of total GLP-1 and active GIP increased after meal intake in all groups. The concentrations of both incretins reached to a maximum value 30 min after meal ingestion. The maximum total GLP-1 level was 23.0 ± 8.7 pmol/l in obese patients, 17.8 ±

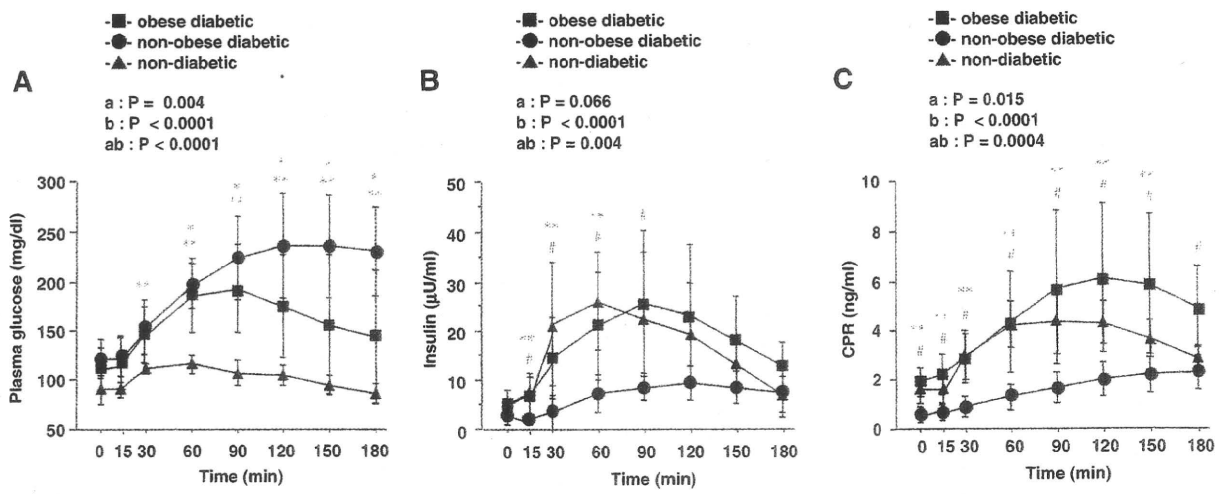

Fig. 1. Results of mixed meal test in (A) glucose, (B) immunoreactive insulin, (C) C-peptide. Data are means ± SD. Statistics were carried out using repeated-measure ANOVA and denote differences between the experiments (a), differences over time (b), and differences due to the interaction of experiment and time (ab). $^{*}P < 0.05$, obese diabetic patients vs non-diabetic subjects. $^{**}P < 0.05$, non-obese diabetic patients vs non-diabetic subjects. $^{\#}P < 0.05$, obese diabetic patients vs non-obese diabetic patients.

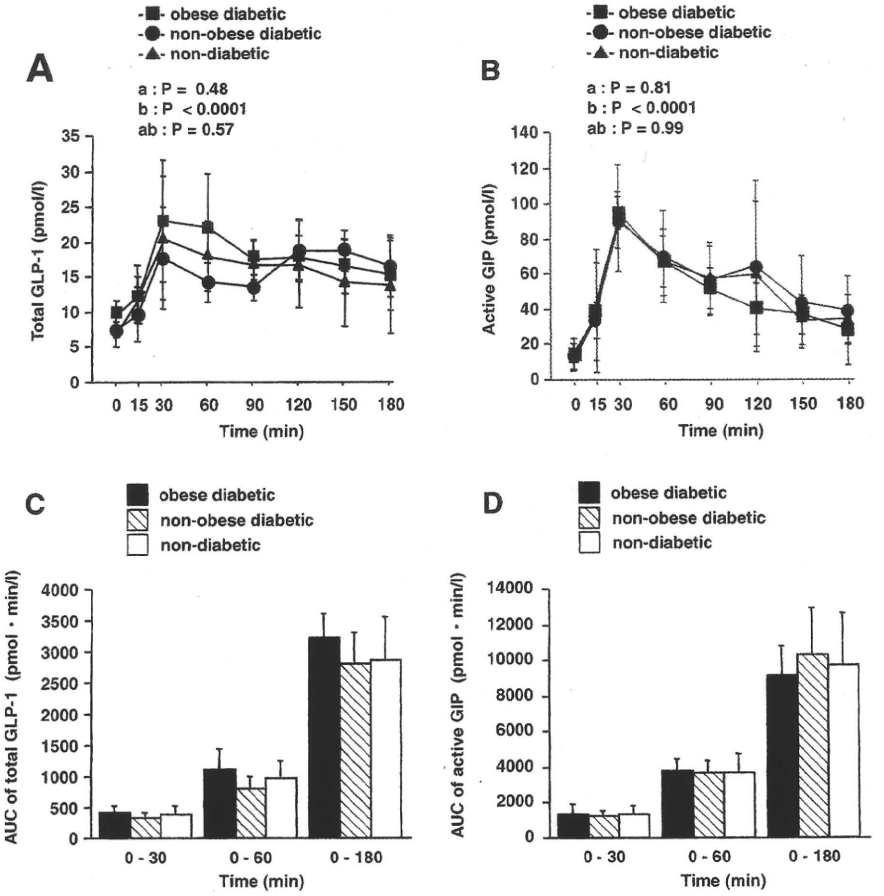

Fig. 2. Results of mixed meal test in (A) total GLP-1, (B) active GIP, (C) AUC of total GLP-1, (D) AUC of active GIP. Data are means ± SD. Statistics were carried out using repeated-measure ANOVA and denote differences between the experiments (a), differences over time (b), and differences due to the interaction of experiment and time (ab).

7.3 pmol/l in non-obese patients and 20.4 ± 8.8 pmol/l in non-diabetic subjects. Total GLP-1 levels showed no significant difference between any two groups through the meal tolerance test (Fig. 2A). The maximum active GIP level was 94.9 ± 9.4 pmol/l in obese patients, 90.6 ± 15.9 pmol/l in non-obese patients and 91.1 ± 30.0 pmol/l in non-diabetic subjects. Active GIP levels also showed no significant difference between any two groups through the meal tolerance test (Fig. 2B). All AUCs of total GLP-1 and active GIP showed no significant difference between any two groups (Fig. 2C and D).

Discussion

In the present study, we have shown that incretin levels did not differ between obese and non-obese patients with type 2 diabetes, even though the number of patients was small. In earlier studies, postprandial GLP-1 secretion in response to oral carbohydrate was considerably attenuated in obese non-diabetic women compared with lean non-diabetic women [13] and combined augmentation of GIP and GLP-1 responses were observed in obese type 2 diabetic patients compared with normal and obese non-diabetic subjects [14]. However, these were very early studies with rather non-specific assays. Our report elucidates incretin levels in Japanese obese and non-obese patients with type 2 diabetes.

Our present results showed a dissociation between the patterns of insulin secretion and incretin secretion in patients with type 2 diabetes. Serum incretin levels were not different between obese and non-obese patients. However, the levels of serum insulin and C-peptide levels were significantly lower in non-obese patients than those in obese patients. Assuming that postprandial incretin secretion is the most responsible for enhancing glucose-stimulated insulin secretion, the dissociation might derive from the loss of incretin effect in non-obese patients with type 2 diabetes. Precise reason for this loss of the effect is not clear, but three possible mechanisms could be considered. The first one is that beta cells lose their ability to react to physiological levels of incretin hormones, which may be called "incretin resistance" as shown both *in vitro* [15] and *in vivo* [16]. The second possible mechanism is that beta cells in obese subjects are relatively more sensitive to incretins compared with in non-obese subjects or stimulated to secrete insulin by other factors than incretins. The last possible mechanism comes from a reduction of beta cell mass. In patients with type 2 diabetes, especially in non-obese patients, beta cell mass is reported to be decreased [17–19]. Reduced insulin secretion in non-obese patients might reflect a reduced beta cell mass in these patients in this study. Among these three possible mechanisms, we think the last one is the most likely, because the fasting C-peptide-to-glucose ratio has a close correlation with beta cell area in humans [20] and this ratio in non-obese patients was significantly lower than that in obese patients in our study.

We also showed similar secretion of incretins among obese diabetic, non-obese diabetic and non-diabetic subjects in Japanese, as partly reported in another Japanese study [21]. The reason for the decreased secretion of GLP-1 in Caucasian subjects with T 2DM is

not known but may be related to insulin resistance and obesity [22]. It is possible that the obesity and insulin resistance in our patients did not reach levels sufficient to influence GLP-1 secretion. The two weeks of treatment prior to the meal test may have improved incretin secretion. Alternatively, it may be that total GLP-1 levels in Japanese subjects are naturally low compared with those in Caucasian subjects [10] and not parallel to insulin secretion.

In this study we measured total GLP-1 (the sum of intact GLP-1 and its primary metabolite GLP-1 9–36 amide), because GLP-1 is degraded almost instantaneously by the enzyme DPP-4 so that the levels of intact GLP-1 do not reflect the L-cell secretion rate. In stead L-cell secretion rate may be estimated by measuring total GLP-1. Since GLP-1 may interact with afferent sensory nerve fibers, locally in the gastrointestinal tract before it is, total GLP-1 levels are also a better measure of the impact of L-cell secretion than the intact levels [23]. GIP is also degraded by DPP-4 but much more slowly, and for GIP the mechanism of action is probably classical endocrine. Therefore, the relevant measure is intact GIP.

In conclusion, incretin secretion did not differ between obese and non-obese patients with type 2 diabetes. The reduced incretin effect observed in Japanese non-obese diabetic patients is therefore attributed to other mechanisms than impaired incretin secretion. In addition, incretin secretion did not differ between diabetic patients and non-diabetic subjects.

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Case Report

Serum 1,5-anhydroglucitol levels in patients with fulminant type 1 diabetes are lower than those in patients with type 2 diabetes

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ABSTRACT

Objectives: We investigated clinical relevance of serum 1,5-anhydroglucitol (1,5-AG) levels in fulminant type 1 diabetes mellitus (FT1DM) patients, because 1,5-AG is known to reflect short term glycemic control.

Design and methods: Subjects comprised 7 patients with FT1DM and 32 patients with type 2 diabetes mellitus (T2DM) with HbA1c <8.5%. All of them have never been treated for diabetes.

Results: HbA_{1C} showed no significant difference between both groups. On the other hand, serum 1,5-AG levels were significantly lower in the FT1DM patients than in the T2DM patients. Serum 1,5-AG levels were<5.0 μg/ml in 6 of 7 (86%) FT1DM patients, compared with only 1 of 32 (3%) T2DM patients.

Conclusions: Serum 1,5-AG levels were lower in the FT1DM patients than in the T2DM patients. Serum 1,5-AG, but not HbA_{1C}, reflects short-term exacerbation of glycemia in patients with FT1DM.

Introduction

Fulminant type 1 diabetes (FT1DM) is a new subtype of type 1 diabetes first reported in 2000 by Imagawa et al. [1] and characterized clinically by acute and almost complete pancreatic β cell destruction without islet-related autoantibodies such as glutamic acid decarboxylase (GAD) antibody or insulinoma-associated antigen 2 (IA-2) antibody [1,2]. In Japanese patients, FT1DM reportedly accounts for about 20% of all cases of type 1 diabetes with abrupt onset [2]. In contrast to plasma glucose (PG) levels, which are markedly elevated at clinical onset, HbA_{1C} levels are normal or only slightly elevated in FT1DM.

It is known that several types of protein glycation increase in diabetic patients compared with non-diabetic subjects. Some of these proteins play roles in the onset and progression of complications in chronic diabetes [3]. Among these glycated proteins, HbA_{1C} is widely used in clinical practice as a marker of glycemic control [4,5]. Since the average lifespan of erythrocytes is about 120 days, HbA_{1C} level reflects glycemic control state over the past 1–2 months. Glycated albumin (GA), as well as HbA_{1C}, has become used as another marker of glycemic control. Since half-life of albumin is shorter than that of erythrocytes, GA reflects short-term glycemic control (about 2 weeks) [6,7]. We have already suggested that GA/HbA_{1C} ratio is high in FT1DM patients, because HbA_{1C} only slightly elevates but GA markedly elevates due to rapid elevation of plasma glucose in these patients [8].

1,5-anhydroglucitol (1,5-AG) is a novel marker of glycemic control in diabetic patients, because urinary excretion of 1,5-AG increases and serum 1,5-AG decreases in the conditions of increased glucose in urine [9,10]. This is because reabsorption of 1,5-AG at kidney is antagonistic to that of glucose. Serum 1,5-AG levels changes faster than HbA_{1C} at the time of rapid deterioration in glycemic control, and serum 1,5-AG reflects postprandial hyperglycemia more correctly than HbA_{1C} [11]. In this study, we examined whether or not serum 1,5-AG reflects short term exacerbation of glycemia in patients with FT1DM.

Methods

Patients studied

Patients of this study were 7 patients with FT1DM whose HbA_{1C} and serum 1,5-AG were determined simultaneously at their initial visit from case reports. Control group consisted of 32 patients with type 2 diabetes (T2DM) whose HbA_{1C} was lower than 8.5%, because inclusion criteria for FT1DM was HbA_{1C}<8.5% at initial visit [1,2], selected from 558 subjects who underwent health examination at Kinki Central Hospital from July to August 2008 (Table 1). They showed no past history of diabetes treatment. Due to potential effects on HbA_{1C} and serum 1,5-AG values, exclusion criteria for both groups were: pregnancy; renal, hepatic, or hematologic disease; and steroid

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Table 1
Clinical characteristics of FT1DM and T2DM.

	FT1DM	T2DM	*p*
n	7	32	
M/F	5/2	23/9	0.981
Age (years)	40.1 ± 13.6	56.0 ± 5.2	<0.0001
BMI (kg/m^2)	20.9 ± 0.5	24.8 ± 3.9	0.0344
PG (mg/dl)	945 ± 297[a]	114 ± 11[b]	-
PG (mmol/l)	52.5 ± 16.5[a]	6.3 ± 0.6[b]	-
HbA_{1C} (%)	5.9 ± 0.7	6.0 ± 0.5	0.7316
1,5-AG (μg/ml)	3.3 ± 1.4	13.5 ± 5.4	<0.0001
Duration from onset (day)	2.0 ± 1.3	-	

[a] Randomly measured plasma glucose.
[b] Fasting plasma glucose.

therapy. Diagnosis of FT1DM and T2DM was based on previously reported criteria. The institutional review board approved this study, and all T2DM patients as control group provided written informed consent.

Laboratory methods

Plasma glucose was determined using the glucose-oxidase method. HbA_{1C} was measured by high performance liquid chromatography (HPLC), with calibration using Japan Diabetes Society (JDS) Lot 2 [12]. Serum 1,5-AG was measured using Lana 1,5AG Auto Liquid Kit using an enzymatic method (Nippon Kayaku, Tokyo, Japan), as described previously [13]. Intra- and inter-assay coefficients of variation for 1,5-AG measurement were 1.7% and 1.2%, respectively.

Statistical analysis

Data are shown as means ± SD. For statistical analyses, unpaired Student's *t*-test was used to compare two groups. To evaluate the relationship between HbA_{1C} and 1,5-AG levels, single linear univariate regression analyses was performed. The Stat View computer program (Version 5.0 for Windows; Abacus Concepts and Berkeley, CA) was used for all statistical analyses. A p value of <0.05 was considered statistically significant.

Results

Table 1 shows clinical characteristics of the study patients. Mean age was significantly younger in FT1DM patients (40.1 ± 13.6 years) than in T2DM patients (56.0 ± 5.2 years; $p<0.0001$). Mean body mass index (BMI) was significantly lower in FT1DM patients (20.9 ± 0.5 kg/m^2) than in T2DM patients (24.8 ± 3.9 kg/m^2; $p=0.0344$). In the FT1DM patients, mean duration from the onset of hyperglycemic symptoms to the start of insulin therapy was 2.0 ± 1.3 days.

In patients with FT1DM at initial diagnosis, randomly measured PG was markedly elevated [945 ± 297 mg/dl (52.5 ± 16.5 mmol/l); range, 509–1195 mg/dl (28.3–66.4 mmol/l)]. In patients with T2DM, fasting PG was 114 ± 11 mg/dl (6.3 ± 0.6 mmol/l) [range, 95–136 mg/dl (5.3–7.6 mmol/l)]. HbA_{1C} showed no significant difference between the groups (5.9 ± 0.7% vs. 6.0 ± 0.5%; $p=0.7316$). On the other hand, serum 1,5-AG levels in the FT1DM patients was significantly lower than that in the T2DM patients (3.3 ± 1.4 μg/ml vs. 13.5 ± 5.4 μg/ml; $p<0.0001$) (Table 1).

In the T2DM patients, there was significant inverse correlation between HbA_{1C} and serum 1,5-AG ($R=-0.621$, $p=0.0002$) (Fig. 1). On the other hand, serum 1,5-AG levels in the FT1DM patients were remarkably low regardless of HbA_{1C} levels. Serum 1,5-AG levels were<5.0 μg/ml in 6 of 7 (86%) FT1DM patients, compared with only 1 of 32 (3%) T2DM patients (Fig. 1).

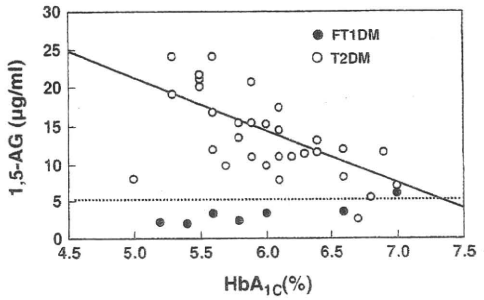

Fig. 1. Relationship between serum 1,5-AG and HbA_{1C}. The figure shows relationship between serum 1,5-AG and HbA_{1C} in the treatment-naive FT1DM patients (closed circle) and the T2DM patients (open circle). The dotted line represents serum 1,5-AG 5.0 μg/ml. The solid line represents the regression line of serum 1,5-AG and HbA_{1C} in the T2DM patients.

Discussion

The diagnosis of FT1DM is clinically important, as initiation of insulin treatment is crucial to avoiding abrupt deterioration and poor prognosis [1,2]. It has been reported that about 10% of FT1DM patients showed randomly measured PG levels ≤ 400 mg/dl (22.2 mmol/l) [14]. By contrast, randomly measured PG in patients with T2DM sometimes exceeds 288 mg/dl (16 mmol/l), which is a diagnostic level for FT1DM. In these cases, FT1DM is not easily distinguishable from T2DM. Clinical indicators other than PG levels are thus needed for differentiating FT1DM from T2DM.

When plasma glucose exceeds threshold of reabsorption at kidney, urinary glucose not reabsorbed suppresses reabsorption of 1,5-AG via sodium glucose cotransporter 4 (SGLT4) at proximal tubules of kidney [9,10]. When glycemic control is improved and urine glucose disappears, the glucose-dependent suppression of 1,5-AG reabsorption disappears, and most of 1,5-AG can be reabsorbed at renal tubules. It is known that pooled serum 1,5-AG is elevated by 0.3 μg/ml/day with 1,5-AG taken from meal [15]. When glycemic control state is poor, urine glucose prevents reabsorption of 1,5-AG, resulting in rapid decrease of serum 1,5-AG. This decrease occurs faster than its increase during improvement of glycemic control, as above [16].

Based on these facts, we investigated whether or not serum 1,5-AG reflects short term exacerbation of glycemia in patients with FT1DM. As far as we have searched, this study is the first report on serum 1,5-AG in FT1DM patients. As a result, serum 1,5-AG levels were lower than the reference level (< 14.0 μg/ml) in all FT1DM patients at initial visit, and markedly low in most of them. When 5.0 μg/ml was used as a cut-off value of serum 1,5-AG, sensitivity was 86% and specificity was 97%, for diagnosis of FT1DM. This finding indicates that serum 1,5-AG, but not HbA_{1C}, reflects short term exacerbation of glycemia in patients with FT1DM.

GA, another glycemic control marker, is known to reflect acute short term changes in plasma glucose, similar to 1,5-AG [6,7]. The rate of change is almost similar for 1,5-AG and GA, when glycemic control is improved. As mentioned, serum 1,5-AG is more sensitive to deterioration than to improvement of glycemic control. Serum 1,5-AG changes faster than GA in FT1DM patients in which plasma glucose elevates rapidly. Thus, it is serum 1,5-AG can reflects shorter term of hyperglycemia than GA in patients with FT1DM. We have already reported that measurement of GA is useful to discern FT1DM from T2DM. When 3.2 is used as a cut-off value of GA/HbA_{1C} ratio, sensitivity was 97% and specificity was 98% for diagnosis of FT1DM [8]. In the present study, sensitivity of serum 1,5-AG was slightly lower than that of GA, contrary to our expectation. The limited number of patients included in this study (7 cases) might have affected the result. It is necessary to examine the utility of 1,5-AG in identifying the

presence of short-term exacerbation of glycemia in patients with FT1DM within an extended number of subjects.

Conflicts of interest statement

None of the authors have conflicts of interest to declare.

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Exendin-4, a glucagon-like peptide-1 receptor agonist, suppresses pancreatic β-cell destruction induced by encephalomyocarditis virus

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ABSTRACT

Viral infection is one of the important factors for the pathogenesis of type 1 diabetes. Particularly, in fulminant type 1 diabetes, rapid β-cell destruction is suggested to be triggered by viral infection. Recently, glucagon-like peptide 1 (GLP-1) receptor agonists have been reported to have direct beneficial effects on β-cells, such as anti-apoptotic effect, increasing β-cell mass, and improvement of β-cell function. However, their effects on β-cell destruction induced by viral infections have not been elucidated. In this study, we used an encephalomyocarditis virus (EMCV)-induced diabetic model mouse to show that a GLP-1 receptor agonist, exendin-4, prevents β-cell destruction. Nine-week-old male DBA/2 mice were intraperitoneally injected with EMCV (200 plaque forming units (PFU) mouse^{-1}). Low (20 nmol kg^{-1} d^{-1}) or high (40 nmol kg^{-1} d^{-1}) doses of exendin-4 were administered for 10 d, starting from 2 d before the infection, and the rate of diabetic onset was evaluated. In addition, the number of infiltrating macrophage per islet and the ratio of β-cell area to islet area were determined. The effects of exendin-4 on infected β-cells and macrophages were investigated by using MIN6 and RAW264 mouse macrophages. The incidence of diabetes was significantly lower in the high-dose exendin-4-treated group than in the control group. Furthermore, the β-cell area was significantly more preserved in the high-dose exendin-4-treated group than in the control. In addition, the number of macrophages infiltrating into the islets was significantly less in the high-dose exendin-4-treated group than in the control group. In vitro, exendin-4 reduced β-cell apoptosis, and tumor necrosis factor α (TNFα), interleukin β (IL-β), and inducible nitric oxide synthase (iNOS) production of infected or lipopolysaccharide (LPS)-stimulated macrophages. These results suggested that exendin-4 limits β-cell destruction by protecting β cells and reducing the inflammatory response of macrophages.

1. Introduction

The onset of type 1 diabetes is strongly associated with genetic factors (such as HLA type) and environmental factors. Particularly, viral infection is regarded as the most important environmental factor [2,3]. In Japan, fulminant type 1 diabetes accounts for about 20% of the cases of acute-onset type 1 diabetes and is characterized by extremely rapid β-cell destruction. Fulminant type 1 diabetes appears to be triggered by viral infection based on the following findings: (1) it occurs after flu-like symptoms; (2) anti-enterovirus antibody titers in these patients are higher than those in healthy individuals [4–6]; (3) macrophages infiltration into islets was detected in autopsy pancreatic tissue from patients who died soon after the onset of fulminant type 1 diabetes; and (4) enteroviral antigen or RNA was detected in some patient's pancreas [7,8].

Encephalomyocarditis virus (EMCV) belongs to the Picornaviridae family and causes diabetes, myocarditis, and paralysis in susceptible mice. After EMCV infection, diabetes occurs within a week as a result of almost complete destruction of β-cells. The clinical course of this model resembles that of fulminant type 1 diabetes in humans [9].

Glucagon-like peptide-1 (GLP-1), a hormone that is secreted from the L-cells of the small intestine during digestion, enhances the glucose-dependent insulin secretion and decreases glucagon secretion [10–12]. GLP-1 receptors are abundantly expressed not only in β-cells but also in many other cells [1]. Recently, it was reported that much higher blood GLP-1 levels than physiological level brought by GLP-1 receptor agonist injection exhibit many effects, including anti-apoptotic and proliferative effects on β-cells [13–16], decreased food intake [22], and protection against heart injuries [23–25].

Here, we show that a high-dose exendin-4, a GLP-1 receptor agonist, suppresses EMCV-induced β-cell destruction through not only anti-apoptotic effect on β-cells but also anti-inflammatory effects against macrophages.

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2. Materials and methods

2.1. Animals

All animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The study protocol was approved by the Animal Care and Use Committee of Osaka Medical College. Male DBA/2 mice 9 weeks old were purchased from CLEA Japan Inc. and housed in bio-safety facilities at Osaka Medical College. Mice were maintained at a constant room temperature of 25 °C with a 12 h photoperiod. They were fed a standard rodent chow and given access to water ad libitum.

2.2. Virus

Encephalomyocarditis virus (EMCV) was kindly provided by Katsuaki Dan (Keio University, Tokyo, Japan). The virus was grown in DBA/2 mice or L929 cells. The viral titer was determined by a plaque assay on L929 cells as described previously [17,18].

2.3. Viral infection and measurement of blood glucose and pancreatic insulin content

Nine-week-old male DBA/2 mice, which are the most susceptible strain to EMCV, were intraperitoneally injected with 200 plaque forming units (PFU) EMCV in 0.1 mL phosphate-buffered saline (PBS) pH 7.4. Low (20 nmol kg^{-1} d^{-1}) ($n = 7$) or high (40 nmol kg^{-1} d^{-1}) ($n = 7$) doses of exendin-4, a GLP-1 receptor agonist, were intraperitoneally injected for 10 d, starting from 2 d before the infection. Control mice ($n = 14$) were intraperitoneally injected with corresponding dose of PBS for same period. Blood samples were obtained from the tail vein and glucose levels were measured by using Glutest Sensor (Sanwa, Japan) for 14 d after infection. Mice with blood glucose levels greater than 250 mg dL^{-1} were considered to be diabetic. Fifteen days after infection, intraperitoneal glucose tolerance test (IPGTT) was performed with 1 g glucose kg^{-1}. Afterwards, the mice were sacrificed to measure the pancreatic insulin content.

Pancreatic insulin content was measured by using the acid ethanol method [28]. Briefly, fresh pancreas was homogenized in acid ethanol (0.18 N hydrochloric acid, 75% ethanol), and then incubated overnight at 4 °C. After centrifugation, the supernatant was neutralized. The insulin concentration of the extract was measured by using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Sibayagi, Japan). The protein concentration was measured by using the bicinchoninic acid (BCA) method. The pancreatic insulin content was calculated by dividing the insulin concentration by the protein concentration.

2.4. Histopathology of the pancreas

Sixteen control mice infected with EMCV and 16 mice treated with high-dose exendin-4 were sacrificed at 48, 72, 96, and 120 h after infection, and then their pancreas was excised, fixed with 4% paraformaldehyde, and embedded in paraffin. Four-micrometer-thick paraffin sections were deparaffinized in xylene and rehydrated in a graded ethanol series (100% to 50%). After washing in PBS, the target retrieval was performed with proteinase K for 6 min. Peroxidase activity was inhibited by immersing the sections in 3% methanolic hydrogen peroxide for 15 min, and non-specific binding of the antibody was blocked by pre-incubation with Non-Specific Staining Blocking Regent (X0909 Dako, Japan). The sections were then incubated at room temperature for 30 min with either rat anti-Mac-2 antibody (CL8942AP Cedarlane Laboratory, 1:500 dilution) or guinea pig polyclonal anti-swine insulin antibody (A0564 Dako, Japan, 1:800 dilution). After washing in PBS 3 times, the slides were incubated with secondary antibodies for 30 min at room temperature; Envision + (K4003 Dako, Japan) was used for the insulin slides and Vectastain Elite ABC Rat Kit (Vector Laboratories, Burlingame, CA) was used for Mac-2 slides. After washing in PBS, the antibody binding was detected by using diaminobenizidine (DAB). Finally, the slides were counterstained with hematoxylin. The β-cell area was measured as the percentage of the insulin-positive area per islet area by using a Scanscope XT Digital Slide Scanner (Aperio). The number of macrophages was counted by using a Scanscope XT Digital Slide Scanner and expressed as the number of Mac-2 positive cells per islet area. At least 5 islets were chosen at random from each section for statistical analysis.

2.5. Flow cytometry apoptosis assay of a β-cell line

MIN6, a β-cell line, was used for the apoptosis assay. MIN6 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 15% fetal calf serum (FCS). Confluent dishes were incubated with EMCV (about 1 PFU $cell^{-1}$) for 48 h or 2 μM staurosporine (Sigma–Aldrich) for 18 h with or without 12 nM exendin-4. In the apoptosis assay with staurosporine, the control wells were incubated with the corresponding concentration of dimethyl sulfoxide (DMSO). After incubation, cell dissociation buffer (Gibco) was added to each well, and then the cell samples were washed with ice-cold medium and centrifuged for 5 min at 500g. Then, the supernatant was thrown away. For the latter process, Annexin V-FITC Kit System for Detection of Apoptosis (Beckman Coulter, Inc., Fullerton, CA) was used. Briefly, samples were diluted with binding buffer to 5×10^5–5×10^6 cells mL^{-1}. Afterwards, the Annexin V-FITC and propidium iodide (PI) solutions were added, and the samples were incubated on ice in the dark for 10 min. Immediately afterwards, the apoptosis rate was measured by flow cytometer, BD FACSAria™ (Becton, Dickinson and Company).

2.6. Exendin-4 pretreatment of LPS activated or EMCV infected macrophages

After washing with PBS, RAW264 macrophage cells were incubated with or without 12 nM exendin-4 for 1 h. Then, EMCV (1 PFU $cell^{-1}$) was added and incubated for 12 h, or 1 μg mL^{-1} LPS (Sigma–Aldrich) was added and incubated for 1 h. To inhibit the exendin-4 signal, RAW264 cells were incubated with 5 μM MDL-12330A (Sigma–Aldrich), an adenylate cyclase inhibitor, at the same time that the exendin-4 was added.

2.7. Isolation of RNA and quantitative real-time polymerase chain reaction

Total RNA was extracted from treated RAW264 cells by using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized by using the Transcriptor First strand cDNA synthesis kit (Roche Diagnostics GMBH, Mannheim, Germany), with the included random hexamers as the priming strategy. The resulting cDNAs were mixed with the LightCycler FastStart DNA Master Hybridization Probes reagent (Roche), TaqMan™ probes, and primers in LightCycler capillaries. Then, the sample carousel with the capillaries was centrifuged in the LightCycler Carousel Centrifuge and placed into the LightCycler. After denaturing for 10 min at 95 °C, 55 polymerase chain reaction (PCR) cycles were run. Each cycle consisted of 10 s at 95 °C and 20 s at 60 °C. Fluorescence was measured at the end of every cycle. After the final cycle, the capillaries were cooled for 2 s at 40 °C. Fluorescence curves were analyzed with the LightCycler software, Ver. 3.5. Cycles 15–55 were used to calculate the crossing points, which were defined

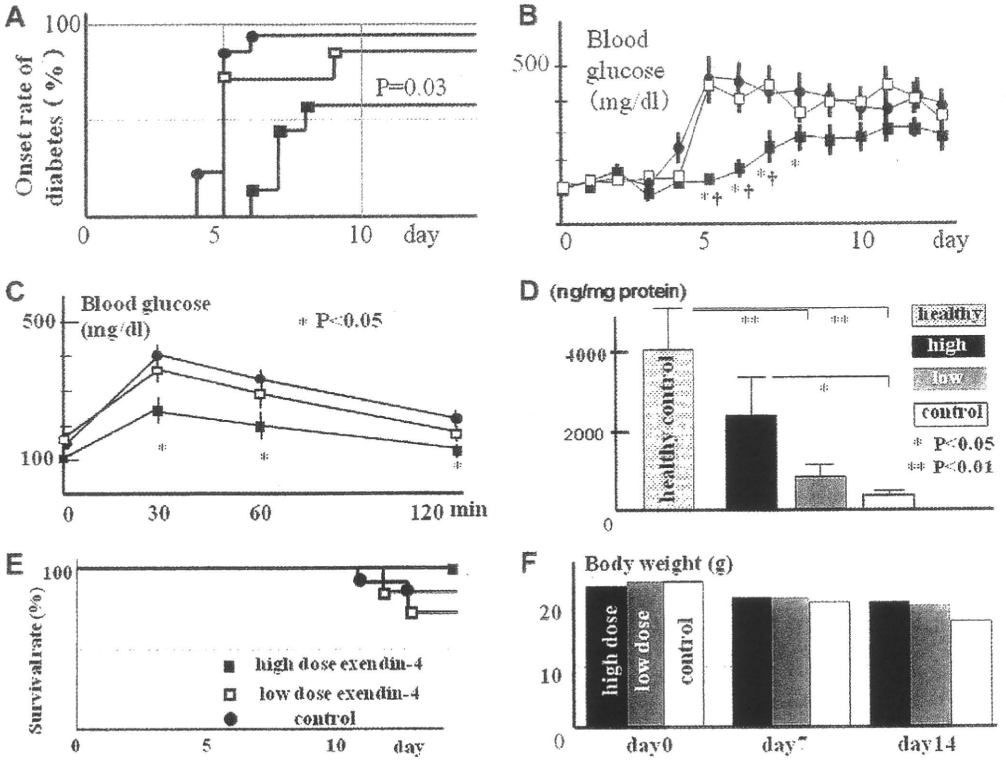

Fig. 1. Exendin-4 prevents the onset of diabetes in encephalomyocarditis virus (EMCV)-infected mice. (A) Changes in the incidence of diabetes during treatment with or without exendin-4 in EMCV-infected mice. ■, High-dose exendin-4; □, low-dose exendin-4; ●, control. $p = 0.03$ control vs. high-dose exendin-4. (B) The change in blood glucose levels after infection. ■, High-dose exendin-4; □, low-dose exendin-4; ●, control. $^{*}p < 0.05$ control vs. high-dose exendin-4, $^{\dagger}p < 0.05$ low-dose exendin-4 vs. high-dose exendin-4. (C) Intraperitoneal glucose tolerance test (IPGTT) 15 d after infection. ■, High-dose exendin-4; □, low-dose exendin-4; ●, control. $^{*}p < 0.05$ control vs. high-dose exendin-4. (D) Pancreatic insulin content 15 d after infection. Black bar, high-dose exendin-4; gray bar, low-dose exendin-4; white bar, control. The bar of healthy control ($n = 4$) was added. (E) Survival rate after infection. ■, High-dose exendin-4; □, low-dose exendin-4; ●, control. There was no significant difference between each group. (F) change in body weight after infection. Black bar, high-dose exendin-4; gray bar, low-dose exendin-4; white bar, control. There was no significant difference between each group.

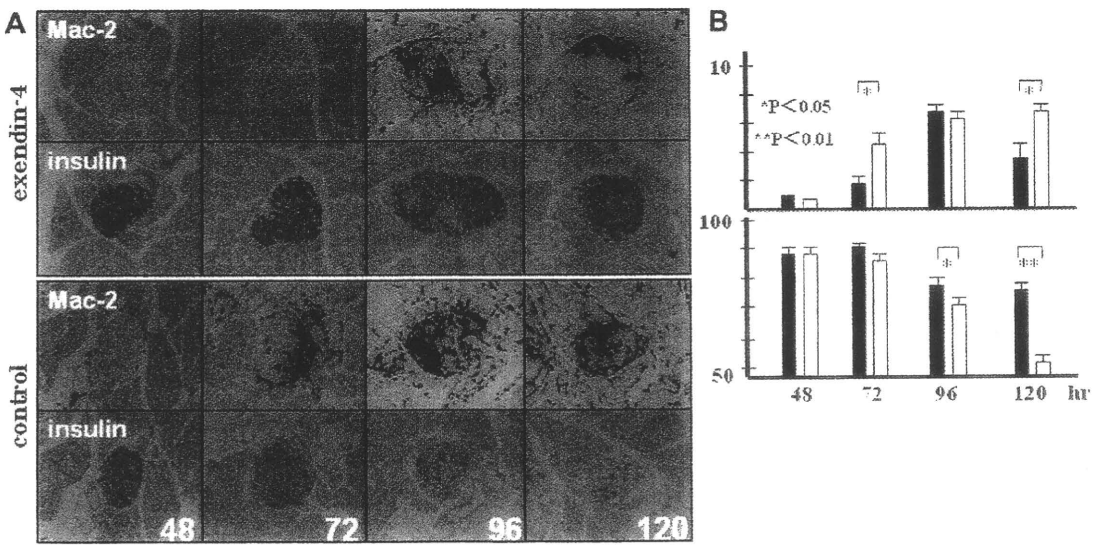

Fig. 2. Exendin-4 reduces macrophages infiltration into islets and β-cell destruction in EMCV-infected mice. (A) Histological changes in the high-dose exendin-4-treated group and control group at 48, 72, 96, and 120 h after infection. Mac-2 positive cells indicate macrophages, and insulin-positive cells indicate β-cells. Original magnification: 150×. (B) The upper panel shows number of macrophages per islet area ($\cdot 10^{-3}/\mu m^2$), and the lower panel shows the (β-cell area/islet area) ×100 in the high-dose exendin-4 group and the control group at 48, 72, 96 and 120 h after infection. Black bar, high-dose exendin-4; white bar, control. Statistical analysis: Student's *t*-test.

as the maximum of the second derivative from the fluorescence curves. Automated calculation was performed by the second derivative maximum method. The sequences of the primers and Roche universal probe number (#) are as follows: TNFα (GenBank

Accession No. M13049.1): left primer (TCTTCTCATTCCTGCTTGT GG), right primer (GGTCTGGGCCATAGAACTGA), and #49 probe; IL-1β (NM_008361.3): left primer (TGTAATGAAAGACGGCACACC), right primer (TCTTCTTTGGGTATTGCTTGG), and #78 probe; iNOS (NM_010927.3): left primer (GGGCTGTCACGGAGATCA), right primer (CCATGATGGTCACATTCTGC), and #76 probe; GAPDH (NM_008084): left primer (TGTCCGTCGTGGATCTGAC), right primer (CCTGCTTCACCACCTTCTTG), and #80 probe.

2.8. Data analysis

The data for the incidence of diabetes and the survival rate were analyzed by the log-rank test. Other data were presented as mean (SEM). Unless noted, statistically significant differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). Sheffe's *F*-test was used for comparing each group. A *p*-value less than 0.05 were considered a statistically significant difference.

3. Results

3.1. The effect of exendin-4 on the prevention of diabetes in EMCV-infected mice

Five days after the infection, the blood glucose level and the incidence of diabetes was significantly higher in the control mice than those in the high-dose exendin-4 treated mice (Fig. 1A and B). IPGTT performed 15 d after infection showed that the glucose levels at 30, 60, and 120 min were significantly lower in the high-dose exendin-4 group than those in the control mice (Fig. 1C). The pancreatic insulin content 15 d after infection was significantly higher in the high-dose exendin-4 group than that in the control group (Fig. 1D). After viral infection, the survival rate was the highest in the high-dose exendin-4 group (Fig. 1E). In addition, after viral infection, the body weight of all of the mice decreased, but the degree of weight reduction was less in the high-dose group than in other groups (Fig. 1F).

3.2. Effects of exendin-4 on β-cell mass and macrophage infiltration of islets

The β-cell area was dramatically decreased in the control group compared with that in the high-dose exendin-4 group, and the degree of macrophage infiltration into the islets was severer in the control group than in the high-dose exendin-4 group (Fig. 2A). To evaluate these findings quantitatively, we measured the percentage of the β-cell area to the area of each islet and the number of macrophages per islet area. After 96 h, the β-cell area was significantly less in the control group than in the high-dose exendin-4 group, and at 72 and 120 h, the number of macrophages was significantly more in the control group than in the exendin-4 group (Fig. 2B).

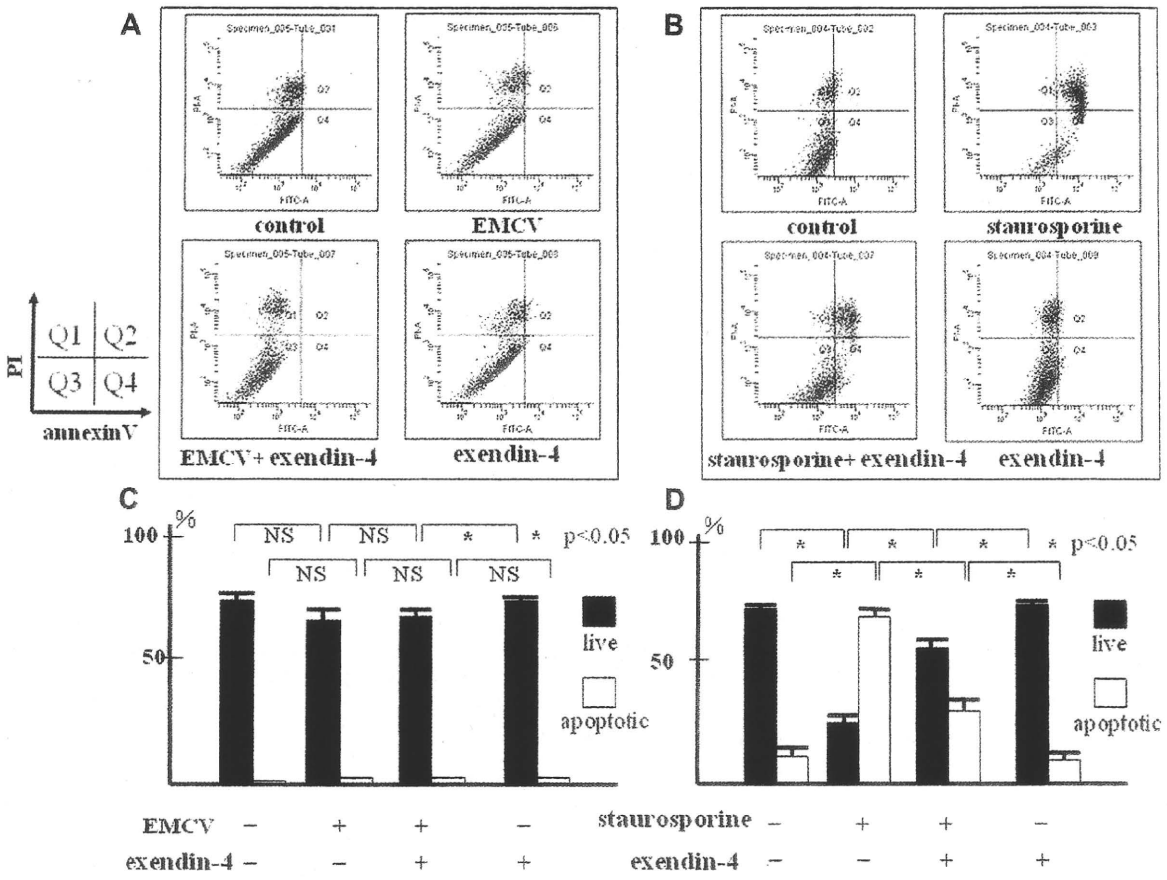

Fig. 3. Exendin-4 has an anti-apoptotic effect on β-cells, but EMCV does not directly induce apoptosis of β-cells. (A) MIN6 cells were incubated for 48 h with EMCV and exendin-4. (B) MIN6 cells were incubated for 18 h with staurosporine and exendin-4. Cells were co-stained with annexin V-FITC and propidium iodide (PI), and then analyzed by flow cytometry. A representative dot-plot is shown for each condition ($n = 3$–5). Q3, live cells; Q4, early apoptotic cells; Q2, late apoptotic cells; Q1, necrotic cells. (C and D) Comparison of the percentage of cells collected as live cells (Q3, black bar) or apoptotic cells (Q2 + 4, white bar) ($n \geq 3$ in each group). Statistical analysis: Student's *t*-test.

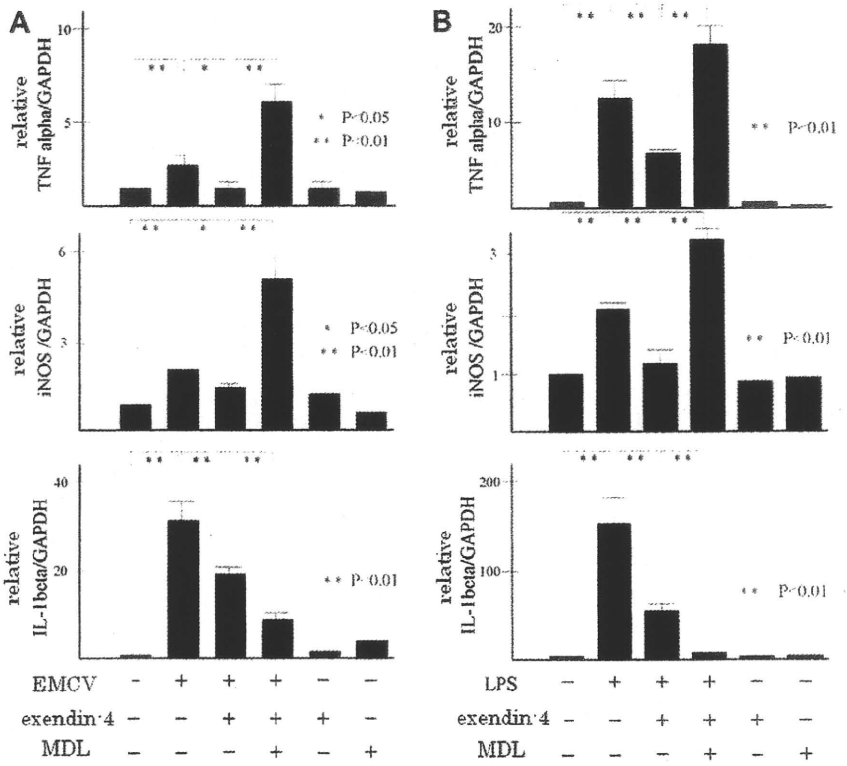

Fig. 4. Exendin-4 reduces the inflammatory response of macrophages. RAW264 cells were incubated with 12 nM exendin-4 for 1 h or with exendin-4 and 5 μM MDL-12330A for 1 h followed by treatment with EMCV (1 plaque forming unit (PFU) $cell^{-1}$) for 12 h (A) or lipopolysaccharide (LPS) (1 μg mL^{-1}) for 1 h (B). Then, the mRNA expression levels of tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and interleukin β (IL-β) were determined by real-time quantitative polymerase chain reaction (RT-PCR). The gene expression level in RAW264 cells without the addition of anything was defined as 1.0 (n = 3–7). Statistical analysis: Student's t-test.

3.3. Exendin-4 protects β-cells from apoptosis, but EMCV does not directly destroy β-cells

To investigate whether exendin-4 has a preventive effect on EMCV-induced β-cell apoptosis, MIN6 cells were incubated with or without EMCV. Forty-eight hours after incubation, no significant difference was observed between the control cells and the EMCV-infected cells (Fig. 3A and C). Staurosporine treatment induced apoptosis in MIN6 cells, but pre-incubation with exendin-4 suppressed it (Fig. 3B and D).

3.4. Exendin-4 modulates macrophage function

Incubation of RAW264 cells with EMCV for 12 h induced a 2–30-fold increases in the RNA expression levels of TNFα, IL-β, and iNOS. Exendin-4 significantly suppressed EMCV-induced RNA expression (Fig. 4A). Likewise, LPS enhanced the RNA expression of TNFα, IL-β, and iNOS, but exendin-4 significantly suppressed them (Fig. 4B). As for TNFα and iNOS, MDL-12330A blocked the effects of exendin-4.

4. Discussion

This study has clearly shown that high-dose exendin-4 injection suppresses the onset of EMCV-induced diabetes. To the best of our knowledge, this is the first report showing that exendin-4 is useful for preventing diabetes induced by EMCV. By inhibiting the infiltration of macrophages into the islets and the expression of macrophage-derived chemical mediators, exendin-4 markedly reduced β-cell destruction. These results suggested that the anti-diabetic effect of exendin-4 occurs through mechanisms that are independent of the enhancement of glucose-dependent insulin secretion or glucagon suppression.

To investigate the mechanism underlying the preservation of β-cells, we first examined whether exendin-4 protects β-cells from EMCV-induced apoptosis. Surprisingly, the in vitro apoptosis assay showed that EMCV did not directly destroy β-cells. On the other hand, exendin-4 suppressed β-cell apoptosis induced by staurosporine, an apoptosis inducer. In mice infected with EMCV, the depletion of macrophages resulted in a much greater decrease in the incidence of diabetes [26,27], which suggested that EMCV did not directly destroy β-cells but macrophage played important role in β-cell destruction.

The infection of macrophages with EMCV induces the expression of chemical mediators [19] and that induce β-cell death [21]. Since macrophages express GLP-1 receptors [20], we hypothesized that chemical mediators, such as TNFα, IL-1β, and iNOS, from infected macrophages play a pivotal role in the destruction of β-cells and that exendin-4 modulates the expression of these mediators. Our results showed that exendin-4 suppressed their expression in infected macrophages. It has previously been reported that EMCV-infected mice treated with an antibody against IL-1β or TNFα or with an iNOS inhibitor exhibited a significant decrease in the incidence of diabetes [30]. Our results are in agreement with this report, which suggested the importance of macrophage-derived mediators in the pathogenesis of diabetes in this EMCV model.

The effect of GLP-1 is mainly mediated by the activation of adenylate cyclase and the elevation of intracellular cyclic AMP levels [29]. By using MDL-12330A, we demonstrated that the

elevation of cyclic AMP levels is essential for the attenuation of TNFα and iNOS mRNA expression in EMCV-infected cells. However, MDL-12330A did not inhibit the suppressive effect of exendin-4 on IL-1β mRNA expression, which indicated that this effect is not mediated by cyclic AMP.

In conclusion, our data suggested that exendin-4 prevents the onset of EMCV-induced diabetes in mice by suppressing the expression of TNF-α, IL-1β, and iNOS in activated macrophages and reducing the β-cell death. Because the macrophages infiltration into islets is observed predominantly in fulminant type 1 diabetes in humans, it has clinical significance to have clarified the effect that exendin-4 suppresses the inflammatory response of activated macrophages. These findings might provide new therapeutic strategies for type 1 diabetes including fulminant type 1 diabetes.

Acknowledgments

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特集：わが国の糖尿病治療においてインクレチン製剤をどのように使うか

2．糖尿病治療の新たな展開ーインクレチンの登場ー

4）DPP-4阻害薬の作用機構と臨床成績

Horikawa Yukio 堀川 幸男　Enya Mayumi 塩谷真由美　Takeda Jun 武田 純

ライフ・サイエンス

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特集 わが国の糖尿病治療において インクレチン製剤をどのように使うか


堀川　幸男

2．糖尿病治療の新たな展開ーインクレチンの登場

4）DPP-4阻害薬の作用機構と臨床成績

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はじめに

DPP-4はポストプロリン-ジペプチジルアミノペプチダーゼ活性をもち，ポリペプチドのN末端から2番目がプロリンかアラニンの場合に，その2つのアミノ酸を切り出すことができる．DPP-4ファミリーには6遺伝子が属し，ほかにFAP，DPP-8，DPP-9，DPL1(DPP-6)，DPL2(DPP-10)が含まれる．DPP-4はユビキタスな多機能のホモ2量体糖タンパクであり，栄養，代謝，免疫，内分泌，造血，癌細胞の増殖，細胞接着，線溶系において重要な役割を果たしている．DPP-4は毛細血管の内皮細胞や活性化リンパ球，また，腺房細胞を含む上皮細胞の管腔側など，すべての臓器に発現している．ヒトにおいては，DPP-4は消化管，胆管，膵臓，腎臓，胸腺，リンパ節，子宮，胎盤，前立腺，副腎，耳下腺，汗腺，唾液腺，乳腺と，肝臓，脾臓，肺，脳を含むすべての臓器の血管内皮に存在しており，細胞膜結合型のほかに，血中可溶型も存在する．DPP-4の結合タンパクには，ADA(アデノシンデアミネース)，腎のNa^+/H^+イオン交換体3，フィブロネクチンなどがある．また，DPP-4の重要な基質には少なくとも9つのケモカイン，NPY，PYY，GLP-1，GLP-2，GIPなどがある．DPP-4阻害薬は，DPP-4によるGLP-1とGIPの不活性化を主に妨害することによって，インスリン分泌を促進し，グルカゴン分泌を抑制し耐糖能の改善効果を示すが，高脂肪食下のDPP-4ノックアウトマウスでは，野生型と比較して食欲は低下し，エネルギー消費が増加することから，DPP-4選択的阻害薬は食欲も抑制し，抗肥満薬としても有用であると考えられる[1,2]．

DPP-4の機能

1．DPP-4と糖脂質代謝

ペプチドホルモンであるGLP-1は糖代謝の重要な調節因子であり，GLP-1はインスリン分泌を促進し，グルカゴン分泌を抑制し，胃内容の排泄を遅延させ食欲低下も来す[3]．しかし，GLP-1受容体ノックアウトマウスでは，糖負荷時のインスリン分泌は低下するものの，グルカゴン値，胃の排泄速度，食事摂取量，体重には変化が認められず，これらの働きは生理的濃度のものではないとも考えられる[4,5]．GLP-1はDPP-4による分解を受けて不活性化され，*in vivo*におけるGLP-1の半減期は2分未満である．また，DPP-4阻害薬は，他の代謝に影響するホルモンであるGIP，VIP，PACAP，GRP(gastrin-releasing peptide)，GLP-2の半減期も延長させる．

DPP-4ノックアウトマウスでは，グルコース投与後のグルコースクリアランスが増加する[6]．非選択的DPP-4阻害薬であるバリン-ピロリジドは，野生型マウスの耐糖能を改善するが，DPP-4ノックアウトマウスでは改善しないことから，耐糖能の改善にはDPP-4がかかわっていることが示唆された．また，バリン-ピロリジドはGLP-1受容体ノックアウトマウスにおいても耐糖能を改善することから，糖代謝の改善すべてがGLP-1に依存する効果ではないことも示唆される[7]．

2型糖尿病モデルであるZucker diabetic fattyラットに長期にDPP-4阻害薬を投与すると，コントロールと

比較して，体重減少と同様に食事誘導性のインスリン分泌が増加する[8]．また，DPP-4ノックアウトマウスでは，高脂肪食誘導性の肥満およびインスリン抵抗性，脂肪肝が発生しにくい．この保護作用は，脂肪酸酸化におけるPPAR-α (peroxisome proliferator-activated receptor-α)の活性化，脂質合成におけるSREBP-1c (sterol regulatory element-binding protein-1c)の非活性化が関係していると考えられているが(表1)[6]，この現象には，脂質代謝に影響するGIPとVIPの半減期延長も関係しているかもしれない．DPP-4阻害薬の空腹時の脂質レベルに対する効果は，あってもわずかであるが，食後の脂質代謝については，臨床的に重要な効果をもつ可能性がある．実際，ビルダグリプチンにより，食後中性脂肪，およびカイロミクロンのアポリポ蛋白であるB-48とカイロミクロンコレステロールを低下させたという報告がある[9]．

DPP-4ノックアウトマウスとラットでは，通常の食事でも野生型より20週齢まで体重が軽い．DPP-4欠損あるいはDPP-4阻害薬による齧歯類の体重減少は，食欲に影響するNPY，GLP-1，PYYと，脂質，炭水化物代謝を調整するPACAP38の増加に由来するかもしれない．肥満の治療を成功させるには，食事摂取と脂肪蓄積の調整メカニズムを同時に標的とすることが重要であり，DPP-4阻害薬の多様な効果はこれらの役割を担うに違いない．一方，DPP-4阻害薬が1型糖尿病の治療に有効かどうかは，研究段階である．

UKPDSが発表されたあと，2型糖尿病の究極的な病理病態学的で生理学的な欠陥，すなわちβ細胞機能の低下を標的とした治療法に大きな注目が集まり，積極的早期介入による強化的な治療法を選択できるような治療法のアルゴリズムが作られた[10,11]．血糖値を低下させ体重減少を来すGLP-1作用には，インスリン分泌促進やグルカゴン分泌抑制など膵島機能(α，β細胞両方)の改善と，おそらく体重減少作用によるインスリン作用(インスリン抵抗性)の改善，両方が含まれる．糖尿病モデルラットでの研究では，ビルダグリプチンとシタグリプチンは膵島細胞量を増加させ，膵島細胞の構造を正常化させる．つまり，DPP-4阻害薬の統合的な薬効は，インスリン分泌促進，グルカゴン分泌抑制，β細胞量の増加により，膵島機能を改善させることである．しかしながら，ヒトにおいては，DPP-4阻害薬によるβ細胞量増加効果が未だ直接証明されていないことは銘記すべきである．また，膵島機能の改善以外に，2型糖尿病患者のインスリン感受性改善効果の可能性も，間接的なインスリン感受性評価と直接的な高インスリン正常血糖クランプテストの両方により示されている．これはDPP-4阻害薬投与による，グルカゴン分泌低下と主に食事摂取量の低下，食後の胃排泄運動抑制によって起こる糖脂質代謝改善によるインスリン作用改善の結果かもしれない(図1)．多面的な効果は未だはっきりと全容が確立されていないが，例えば心血管に直接作用する血糖降下薬として，糖尿病治療における新次元を今後開く可能性もあるのである．

表1　DPP-4欠損マウスの表現型

	野生型マウス	DPP-4欠損マウス
体　重	↑	
食餌摂取量	↑	↓*
熱産生		↑
血中インスリン	↑↑	↓*
血中GLP-1		↑*
血中レプチン	↑↑	↑
脂肪細胞のサイズ	↑	
肝内脂肪	↑	
肝内SREBP		↓
肝内PPAR-α		↑
膵島過形成	あり	なし
インスリン抵抗性	あり	なし
ストレプトゾトシン誘発性高血糖とβ細胞欠損	あり	なし

*高脂肪食下のDPP-4欠損マウス/野生型マウス，他は高脂肪食下/低脂肪食下．　(文献6より引用改変)

このほか，DPP-4阻害薬と免疫，癌，凝固・線溶系，血圧，血管新生との関連なども報告[12]されているが，他稿に譲りここでは省略する．

DPP-4阻害薬の実際

シタグリプチンはアメリカFDAにより2006年10月に認可され，2007年4月にEMEA(European Medicines Agency)に認可された．シタグリプチンはメトホルミン，チアゾリジン系，スルフォニルウレア薬(SU薬)，との併用が認可されており，推奨用量は100 mg/日である．ビルダグリプチンは2008年2月にEMEAから，メトホルミン，チアゾリジン系，SU薬との併用が認可されており，メトホルミン，チアゾリジン系と併用する場合には50 mgを1日2回，SU薬と併用する場合には，50 mgを1日1回で推奨されている．

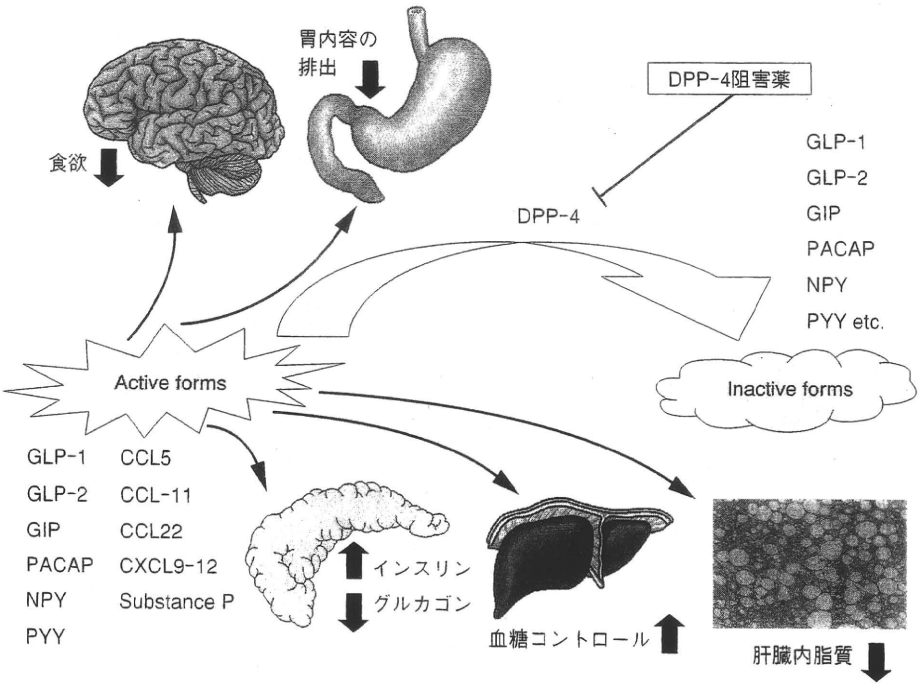


図1　DPP-4阻害薬の糖代謝改善作用

（文献12より引用改変）

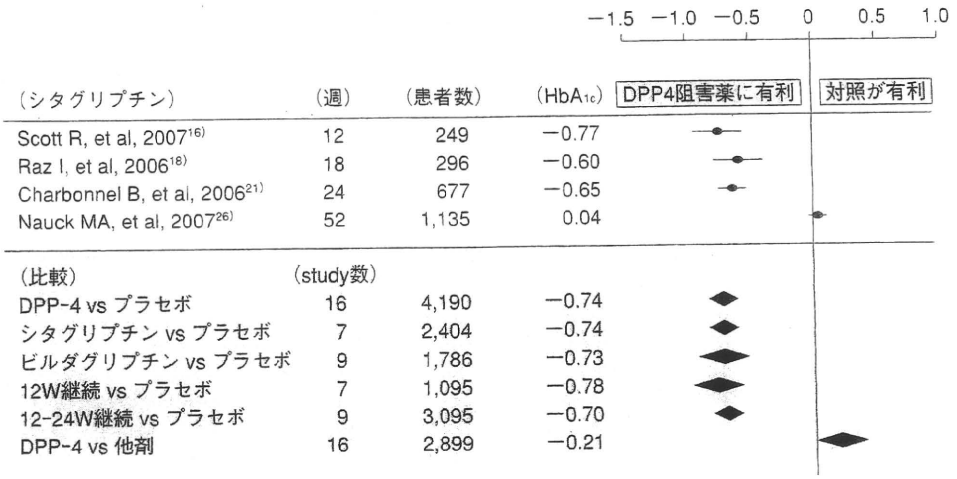
アメリカでは，2007年2月にFDAはビルダグリプチンを認可したが，政府規制機関は腎不全患者における効果について，追加データの発表を待っている．さらに，ビルダグリプチンとメトホルミンの合剤が2008年2月にEMEAに承認されたが，シタグリプチンとメトホルミンの合剤も2008年7月にEMEAに承認されている．これらDPP-4阻害薬は経口摂取後すぐに吸収され，最高血中濃度を1～2時間以内に示し，80%以上が生物学的活性を有する．DPP-4阻害作用発現は早く，経口内服後ほぼ100%の阻害作用が30分以内に観察される．酵素阻害作用時間は量依存的で，臨床的な量の場合，60～80%以上の阻害効果が24時間継続する．

DPP-4阻害薬の代謝は，それぞれ異なる．シタグリプチンは主に腎臓より，未変化体のまま排泄される．したがって腎不全時には，シタグリプチンの血中濃度が上昇する．ビルダグリプチンは80%が加水分解され，不活性化されたあと尿中に排泄される．20%のみがそのまま排泄される．DPP-4阻害薬と他の薬剤との薬物代謝相互作用はこれまで観察されていない．

DPP-4阻害薬の臨床効果

活性型GLP-1の生物学的活性を調節するユビキタスな発現酵素であるDPP-4阻害薬には，現在ビルダグリプチン，シタグリプチン，サクサグリプチン，アログリプチンなどがある．これらの製剤のうち2つ(シタグリプチンとビルダグリプチン)が既に臨床応用されており，シタグリプチンはアメリカとヨーロッパで認可されており，ビルダグリプチンはヨーロッパでの使用が認可されている．

DPP-4阻害薬は，単剤あるいは他の経口薬(SU薬，チアゾリジン系，ビグアナイド系)との併用で，HbA_{1c}を平均約0.5～0.8%低下させるが，臨床上の効果はGLP-1アナログに比べて多少小さい．DPP-4阻害薬は食欲や食事摂取量の抑制効果も報告されておらず，単独で体重をほとんど変化させず(－0.2～＋0.8 kg)，血糖値を低下させるが，臨床スタディの結果では，DPP-4阻害薬はメトホルミンとの併用で血糖低下作用が強くなることが示されている．多くの研究では，DPP-4阻害薬は体重変化を来さないとしており，チアゾリジン系，SU薬，インスリンなどの体重増加作用に対して利点を有する．しかしながら，体重変化を来さないという点は，GLP-1アナログが体重減少を来すのと明らかに異なる．おそらく，DPP-4阻害薬とGLP-1アナログによるGLP-1の血中濃度の違いに由来すると考えられる．DPP-4阻害薬とGLP-1アナログの併用について報告されたデータは未だない．



(シタグリプチン)	(週)	(患者数)	(HbA1c)
Scott R, et al, 2007[16]	12	249	−0.77
Raz I, et al, 2006[18]	18	296	−0.60
Charbonnel B, et al, 2006[21]	24	677	−0.65
Nauck MA, et al, 2007[26]	52	1,135	0.04
(比較)	(study数)		
DPP-4 vs プラセボ	16	4,190	−0.74
シタグリプチン vs プラセボ	7	2,404	−0.74
ビルダグリプチン vs プラセボ	9	1,786	−0.73
12W継続 vs プラセボ	7	1,095	−0.78
12-24W継続 vs プラセボ	9	3,095	−0.70
DPP-4 vs 他剤	16	2,899	−0.21

図2　DPP-4阻害薬による臨床研究でのHbA1cの変化
(文献34より引用改変)

DPP-4阻害薬の臨床スタディ

2型糖尿病患者に，DPP-4阻害薬を単独，あるいは併用で使用することで，血糖コントロールを改善したというスタディがいくつか報告されている(図2)．

1．単独療法[13-20]

DPP-4阻害薬のビルダグリプチン，シタグリプチン，サクサグリプチン，アログリプチン単剤治療のスタディにおいては，ほとんどのスタディで，最短12週間投与でHbA1c 0.5〜1.1%の低下がみられている．HbA1cの改善は1年間継続しており，またHbA1cの投与前値が高いほど，効果も高くなっている．また，年齢や肥満度による効果の相違は認められていない．2型糖尿病患者における単剤治療として，他剤と効果を比較するプロトコールにおいても，DPP-4阻害薬はメトホルミンやチアゾリジン系と同等の効果が示されている．

2．他剤とのコンビネーション治療におけるDPP-4阻害薬

1）メトホルミンとの併用における他剤との比較[21-26]

ビルダグリプチン，シタグリプチン，サクサグリプチン，アログリプチンはいずれも，血糖コントロール不良な患者におけるメトホルミンとの併用について，大規模なスタディが行われている．これらではすべて有効性が認められ，有意なHbA1c改善効果が認められた．4剤の間にはっきりとした差はみられない．しかしながら，各DPP-4阻害薬を相互に直接比較するスタディは未だ行われていない．

2）チアゾリジン系との併用[27]

DPP-4阻害薬とチアゾリジン系を併用するスタディでも，血糖コントロール改善が認められている．

3）SU薬との併用[28]

DPP-4阻害薬とSU薬を併用するスタディでも，血糖コントロール改善作用が認められている．低血糖の増加の有無に関しては以下で述べる．

4）インスリンとの併用[29]

DPP-4阻害薬は，インスリン治療中の2型糖尿病患者に併用した場合も有効である．しかも低血糖のリスクを減少させられる可能性がある．

5）メトホルミン+スルフォニルウレア薬またはチアゾリジン系との併用[30]

DPP-4阻害薬を含む，3剤併用した場合のスタディも行われている．少なくとも6カ月間のスタディにおいては，血糖コントロールの有効な改善効果がみられている．HbA1cは平均0.6〜1%改善し，HbA1cが高い場合ほど効果が高い．

日本人では，シタグリプチン単独療法12週でHbA1cが1.1%減少したのに対して，欧米人では18週で0.79%と，日本人でより強い血糖降下作用が認められている[17,19]．したがって単独投与の場合，血糖降下作用の強さから考え，DPP-4阻害薬はHbA1c 8%以下とあまり高くない患者に使い，6.5%未満という治療目標の達成を目指すのが適切と考えられる．

経口薬のDPP-4阻害薬は，メトホルミンとのコスト差の問題さえクリアできれば，日常診療で軽症糖尿病治療の第一選択薬になる可能性がある(図3)．また，ピオグリタゾン，メトホルミン，SU薬のいずれとも，シタグリプチンの併用で12週にて血糖改善効果が認められている．しかし，SU薬との併用は低血糖を起こす