ORIGINAL

Addition of sitagliptin or metformin to insulin monotherapy improves blood glucose control *via* different effects on insulin and glucagon secretion in hyperglycemic Japanese patients with type 2 diabetes

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Abstract. This study aimed to explore the effects of the dipeptidyl peptidase-4 inhibitor sitagliptin and the biguanide metformin on the secretion of insulin and glucagon, as well as incretin levels, in Japanese subjects with type 2 diabetes mellitus poorly controlled with insulin monotherapy. This was a single-center, randomized, open-label, parallel group study, enrolling 25 subjects. Eleven patients (hemoglobin A1c [HbA1c] $8.40 \pm 0.96\%$) and 10 patients ($8.10 \pm 0.54\%$) on insulin monotherapy completed 12-week treatment with sitagliptin (50 mg) and metformin (750 mg), respectively. Before and after treatment, each subject underwent a meal tolerance test. The plasma glucose, glucagon-like peptide-1 (GLP-1), glucose-dependent insulintropic polypeptide (GIP), C-peptide, and glucagon responses to a meal challenge were measured. HbA1c reductions were similar in patients treated with sitagliptin ($0.76 \pm 0.18\%$) and metformin ($0.77 \pm 0.17\%$). In the sitagliptin group, glucose excursion during a meal tolerance test was reduced and accompanied by elevations in active GLP-1 and active GIP concentrations. C-peptide levels were unaltered despite reduced glucose responses, while glucagon responses were significantly suppressed ($-7.93 \pm 1.95\%$ of baseline). In the metformin group, glucose excursion and incretin responses were unaltered. C-peptide levels were slightly increased but glucagon responses were unchanged. Our data indicate that sitagliptin and metformin exert different effects on islet hormone secretion in Japanese type 2 diabetic patients on insulin monotherapy. A glucagon suppressing effect of sitagliptin could be one of the factors improving blood glucose control in patients inadequately controlled with insulin therapy.

Key words: Insulin monotherapy, Sitagliptin, Metformin, Glucagon, Incretins

FUNCTIONAL deterioration and reduced pancreatic islets of Langerhans mass are the hallmarks of type 2 diabetes mellitus. Incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), modulate islet hormone secretion. Both GLP-1 and GIP augment glucose-stimulated insulin secretion by enhancing the glucose-initiated signaling in islet β -cells [1]. GLP-1 also suppresses glucagon secretion from islet α -cells [1]. Effects of GIP on glucagon secretion are, however, dependent on prevailing glucose concentrations: this hormone stimulates glucagon secretion during periods of euglycemia,

but has little or no effect on glucagon release when glucose concentrations are elevated [2, 3]. In contrast to effects on insulin secretion, the precise mechanisms by which incretins regulate glucagon secretion are not well established [4]. In subjects with type 2 diabetes, secretion of incretins appears to be largely unaltered [5, 6]. The insulintropic effect of GLP-1 is somewhat reduced yet preserved [2], while that of GIP is essentially absent in type 2 diabetes [2, 7, 8]. In addition, although GLP-1 suppresses glucagon secretion, GIP infusion has been demonstrated to antagonize the glucagonostatic effects of GLP-1 in type 2 diabetes [8].

Dipeptidyl peptidase-4 (DPP-4) inhibitors prevent degradation of incretin hormones and augment their activity in the blood stream. In type 2 diabetes patients managed with diet or oral hypoglycemic agents, DPP-4 inhibitors were shown to improve blood glucose control by increasing β -cell responsiveness and suppress-

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ing glucagon secretion [9-12]. Although these drugs were also demonstrated to ameliorate poor blood glucose control in insulin-treated type 2 diabetes patients [13-16], few studies have reported on blood glucose lowering mechanisms, especially in East Asian populations. In patients on insulin monotherapy, peripheral blood insulin levels are sufficient. Thus, DPP-4 inhibitors are thought to exert their blood glucose lowering effects *via* mechanisms other than increasing peripheral insulin levels. A glucagon suppressive effect is one of these possible mechanisms. However, since DPP-4 inhibitors augment plasma activities of both GLP-1 and GIP, the latter being capable of antagonizing the glucagonostatic effect of the former [8], it is not clear whether DPP-4 inhibitors cause glucagon suppression in hyperglycemic insulin-treated type 2 diabetes patients.

Metformin is a member of the biguanide class of oral anti-hyperglycemic agents. Several studies, though not all, have demonstrated metformin to increase active GLP-1 levels in diabetic patients treated with oral agents [17-20]. Addition of metformin to the treatment regimens of patients on insulin monotherapy was shown to improve blood glucose control [21]. There is, however, little information as to metformin effects on glucagon secretion and incretin hormone levels in insulin-treated patients.

In this study, therefore, we aimed to explore the effects of sitagliptin and metformin on the secretion of insulin and glucagon, as well as incretin levels, in subjects with type 2 diabetes poorly controlled on insulin monotherapy. For this purpose, we have designed a parallel group study in which patients on insulin monotherapy had been recruited and responses of these hormones to a standard meal were analyzed after 12 weeks of therapy with the two drugs.

Materials and Methods

Study design and participants

This study is a single-center randomized open-label parallel group study, designed in accordance with the principles stated in the Declaration of Helsinki, and conducted at Nihon University Itabashi Hospital between June 2012 and May 2013. The original study protocol was reviewed and approved by the Institutional Review Board of Nihon University Itabashi Hospital and registered with the clinical trial registry of the

University Hospital Medical Information Network (UMIN 000008155). Written informed consent was obtained from all participants.

We enrolled 25 Japanese patients treated with insulin but without oral anti-diabetes agents, and randomly allocated them to either sitagliptin (50 mg before breakfast) or metformin (250 mg before every meal), using the minimization methods by which distributions of sex, basal hemoglobin A1c (HbA1c), and insulin doses per body weight were balanced. The randomization was conducted by an independent company.

Other key inclusion criteria were: 1) confirmed diagnosis of type 2 diabetes, 2) no changes in treatment for the prior three months, 3) HbA1c of at least 6.9% but no more than 10.5%, 4) more than 20 but less than 75 years of age, and 5) submission of a completed consent form for participation in this study. Key exclusion criteria were 1) diagnosis of type 1 diabetes, 2) glucagonoma, 3) being treated with oral hypoglycemic agents, 4) nephrotic syndrome, 5) being treated with steroids, immune-suppressive agents and/or azole antifungal medication, 6) allergies to sitagliptin and/or metformin, 7) renal impairment (serum creatinine, men: equal to or greater than 1.3 mg/dL, women: equal to or greater than 1.2 mg/dL), 8) liver dysfunction (AST, ALT greater than 3 times upper standard values), and 9) being pregnant, breast-feeding and/or having the intention to become pregnant.

All but 2 patients had been treated with rapid insulin before every meal and with long-acting basal insulin analogues either before breakfast or at bedtime. One patient had been prescribed rapid-acting insulin before all three meals without basal insulin, and another rapid-acting insulin before breakfast and lunch as well as biphasic insulin before supper. Insulin doses had been fixed throughout the study period unless hypoglycemic events occurred, in which case attending physicians had the responsibility of deciding whether doses should be changed.

Meal tolerance test

Meal tolerance tests (MTT) were performed immediately before administration of sitagliptin or metformin and after 12-week treatments with either of these oral agents. The patients were given a standard test meal [22] (Q.P. Corporation, Tokyo, Japan) at 9:00 a.m. This meal consists of 5 crackers, a cup of pudding, and a cup of chicken cream stew and contains a total energy of 450 kcal with 51.4% from carbohydrates, 33.3%

from fats and 15.3% from proteins. Before ingesting the test meal, patients injected insulin as usual, took sitagliptin or metformin and had the meal within 10 minutes. Then, the first blood sample was drawn at time zero. Blood was drawn into chilled tubes containing EDTA and aprotinin (500 KIU/mL blood) and the samples were then kept on ice. After centrifugation at 4°C, plasma samples for hormone assay were divided into aliquots of 0.5 ml and stored at -80°C.

Laboratory determinations

HbA1c was assayed using high-performance liquid chromatography (Tosoh, Tokyo, Japan). Plasma glucose was measured by the hexokinase method. Blood biochemical data were obtained employing a standard laboratory assay. Plasma glucagon concentrations were determined with Linco radioimmunoassay (RIA) kits (Millipore, Temecula, CA, USA). Plasma concentrations of active GLP-1, active GIP, and total GIP were measured using ELISA kits (Immuno-biological Lab. Takasaki, Japan). Total GLP-1 was measured using an ELISA kit from Yanaihara Institute Inc. (Fujinomiya, Japan), which has high specificity to all three amidated isoforms and shows low cross-reactivity to non-amidated forms. We did not perform extraction of plasma samples before measurements using these kits. It has been proposed that active GLP-1 levels should be measured after ethanol extraction or solid-phase extraction because unknown interference could yield higher and variable values of active GLP-1 [23]. Another paper also recommended extraction for accurate measurement [24]. We appreciate these proposals and believe that absolute concentrations of active GLP-1 reported in this study should not be compared with those in other studies. We have, however, thought measurement without extraction was sufficient to examine whether active GLP-1 levels were increased or not with sitagliptin treatment in this study. The reason is that we have found a good correlation between active GLP-1 concentrations assayed with and without ethanol extraction using the active GLP-1 ELISA kit from the Immuno-biological Lab ($r = 0.9065$, $P < 0.0001$, $n = 40$).

Calculations and statistical methods

The area under the curve (AUC) was calculated for the response above zero over 120 min obtained by MTT using the trapezoidal rule. Patient characteristics are reported as means $\pm$ SD, and results obtained by MTT are expressed as means $\pm$ SEM. Statistical analyses

were performed using JMP version 10.0 software (SAS Institute, Cary, NC, USA). Differences between values at baseline and those after 12-week treatment in each group were assessed using the Wilcoxon signed-rank test. Differences in the change from baseline between two treatment groups were analyzed using ANCOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Twenty-five subjects (10 women) were enrolled in this study. Two patients withdrew consent to participate because of gastrointestinal discomfort. One patient withdrew because she was unable to eat the test meal. One was unable to visit our hospital due to his family changing residence. Thus, these four subjects were excluded from those who completed the study. The characteristics of the 11 who completed sitagliptin treatment and 10 who completed metformin treatment are presented in Table 1. None of these parameters differed between sitagliptin and metformin groups. These patients had shown poor control of diabetes on insulin monotherapy with average HbA1c levels greater than 8.0%, despite multiple daily insulin injections with average total doses amounting to more than 0.5 units/kg body weight.

After the addition of sitagliptin (50 mg/day) to the preexisting treatment regimens for 12 weeks, body weights were unaltered, but HbA1c (Fig. 1A) and glycated albumin had decreased by $0.76 \pm 0.18\%$ ($P = 0.022$) and by $3.2 \pm 0.7\%$ ($P = 0.003$), respectively. No patients experienced hypoglycemia and insulin doses were not changed. Fasting plasma glucose concentrations did not change after treatment, but glucose excursion during the MTT was decreased (Fig. 1B). Thus, the glucose AUC was lower (412.6 ± 30.0 mg·h/dL) after 12-week treatment than that before sitagliptin treatment (511.6 ± 34.5). Active GLP-1 concentrations in the fasting state tended to be higher ($P = 0.074$) and were significantly increased after the meal challenge, showing GLP-1 AUC to be 47% greater (43.2 ± 9.0 pmol·h/L) than before sitagliptin treatment (29.2 ± 4.2) (Fig. 1C). Active GIP concentrations also increased significantly in response to the meal challenge, from 78.2 ± 11.6 pmol·h/L at baseline to 157.5 ± 18.7 (Fig. 1D) after 12-week treatment. In contrast, total GLP-1 and total GIP levels were lower by 27.4% ($P = 0.017$, Fig. 1E) and 26.4% ($P = 0.007$, Fig. 1F), respectively,

Table 1 Demographic and baseline characteristics of the completers population

	Sitagliptin (N = 11)	Metformin (N = 10)
Female	5	4
Age (years)	65.5 ± 8.7	58.9 ± 11.5
BW (kg)	62.9 ± 11.0	59.6 ± 14.0
Body mass index (kg/m ²)	24.0 ± 3.2	22.4 ± 3.6
Duration of diabetes (years)	14.5 ± 7.5	20.7 ± 9.1
Duration of insulin therapy (years)	7.9 ± 6.2	11.9 ± 6.0
Total Insulin dose (units/day)	31.2 ± 9.6	31.9 ± 14.3
Long-acting insulin (units/day)	10.6 ± 5.6	12.1 ± 6.8
Rapid-acting insulin (units/day)	20.7 ± 6.9	19.8 ± 9.9
Long-acting/total insulin ratio	0.34 ± 0.12	0.38 ± 0.14
Insulin injections		
4 times/day	10	9
3 times/day	1	1
Incidence of complications (person)		
Neuropathy	4	4
Retinopathy	9	7
Macroalbuminuria	2	3
Microalbuminuria	4	2
Estimated GFR (ml/min/1.73m ²)	82.3 ± 29.0	87.7 ± 35.1
Fasting plasma glucose (mg/dL)	193 ± 60	170 ± 46
Fasting plasma C-peptide (ng/mL)	1.32 ± 0.53	1.08 ± 0.88
Fasting plasma glucagon (pmol/L)	19.8 ± 6.4	20.5 ± 6.7
HbA1c (%)	8.40 ± 0.96	8.10 ± 0.54
Glycated albumin (%)	24.0 ± 1.6	23.0 ± 1.2

Data are expressed as Means ± SD.

as compared to those at baseline. C-peptide responses during MTT did not change after sitagliptin treatment (Fig. 1G). Judging from the C-peptide over glucose ratios, however, the data suggest β -cell responsiveness to be improved by sitagliptin in these patients. Glucagon elevations after the meal challenge were significantly suppressed (Fig. 1H), from 47.4 ± 4.4 pmol·h/mL to 43.6 ± 4.2 . Eight of 11 patients showed decreased glucagon AUC while 3 showed no changes in this parameter (Fig. 1I). In addition to the data presented above, we measured plasma lipid levels, proinsulin over C-peptide ratios, plasma creatinine and liver enzyme concentrations. None of these values changed after adding sitagliptin (data not shown).

After the addition of metformin (750 mg/day) for 12 weeks, body weights were unchanged but HbA1c (Fig. 2A) and glycated albumin had decreased by $0.77 \pm 0.17\%$ ($P = 0.022$) and by $2.9 \pm 0.5\%$ ($P = 0.005$), respectively. Four patients experienced hypoglycemia, and average insulin doses were reduced to 28.6 ± 13.9 units/day. Neither fasting plasma glucose

concentrations nor glucose excursion during MTT changed (Fig. 2B). In contrast to the sitagliptin effects described above, neither active nor total forms of incretins changed significantly (Fig. 2C-F). We found that the C-peptide response during MTT increased slightly after metformin treatment (Fig. 2G), 4.05 ± 0.94 ng·h/mL to 4.67 ± 0.98 ($P = 0.021$). Although glucagon levels at time zero tended to increase ($P = 0.059$), glucagon AUC values during meal loading were unchanged (Fig. 2H). Individual glucagon responses varied, with 4 patients showing decreases, 4 increases and the other 2 no changes in glucagon AUC (Fig. 2I). Proinsulin over C-peptide ratios, plasma creatinine and liver enzyme concentrations were unaltered after metformin addition (data not shown).

Since addition of sitagliptin or metformin resulted in similar glycemic improvements (Fig. 3A and 3B), we compared the effects of these drugs on incretin responses and islet hormone secretion. Increases in active GLP-1 AUC tended to be greater with the addition of sitagliptin as compared to metformin (Fig. 3C). Increases in active GIP levels were observed only in patients treated with sitagliptin (Fig. 3D). Total GLP-1 AUC significantly decreased by sitagliptin and tended to increase by metformin (Fig. 3E). Total GIP AUC decreased by sitagliptin and tended to decrease by metformin. The between-group difference did not reach statistical significance (Fig. 3F). Glucose responsiveness of β cells, as estimated by CPR over glucose ratios, was equally improved by these drugs (Fig. 3G). The reduction in glucagon AUC with sitagliptin was significantly greater than that with metformin treatment (Fig. 3H).

Discussion

We studied the effects of sitagliptin and metformin in Japanese patients with type 2 diabetes not adequately controlled with insulin monotherapy. Since Asian diabetic patients account for more than 60% of the world's diabetes population and likely have features of islet function different from those of Caucasians [25], it is important to collect precise information about how these drugs affect islet hormone secretion and incretin levels in Japanese patients treated with insulin. The study subjects all had relatively long disease durations and their islet function had likely deteriorated markedly. Fasting C-peptide is reportedly reduced in Japanese type 2 diabetes patients, decreasing with dia-

Fig. 1 Effects of sitagliptin on HbA1c as well as blood glucose, incretins, insulin and glucagon during meal challenge in type 2 diabetes subjects on insulin monotherapy.

(A) Time course of HbA1c. (B – H) At week 0 (open circles) and week 12 (closed circles), subjects took sitagliptin, injected rapid acting insulin and consumed a test meal; blood was then drawn for measurement of blood glucose (B), active GLP-1 (C), active GIP (D), total GLP-1 (E), total GIP (F), C-peptide (G), and glucagon (H). Individual AUC of glucagon responses are presented in (I). The AUC was calculated for the response above zero over 120 min. P values in B – H were calculated by comparing AUC. Data in A – H are presented as means $\pm$ SEM.

Fig. 2 Effects of metformin on HbA1c as well as blood glucose, incretins, insulin and glucagon during meal challenge in type 2 diabetes subjects on insulin monotherapy.

(A) Time course of HbA1c. (B – H) At week 0 (open circles) and week 12 (closed circles), subjects took metformin, injected rapid acting insulin and consumed a test meal; blood was then drawn for measurement of blood glucose (B), active GLP-1 (C), active GIP (D), total GLP-1 (E), total GIP (F), C-peptide (G), and glucagon (H). Individual AUC of glucagon responses are presented in (I). The AUC was calculated for the response above zero over 120 min. *P* values in B – H were calculated by comparing AUC. Data in A – H are presented as means $\pm$ SEM.

Fig. 3 Comparison of changes of glycemic control, incretins and islet hormone responses between patients with sitagliptin or metformin added to their insulin treatment regimens.

Changes between before and the end of treatment with sitagliptin (closed bars: ■) or metformin (hatched bars: ▨) were calculated for HbA1c (A), glycated albumin (B), active GLP-1-AUC during MTT (C), active GIP-AUC (D), total GLP-1-AUC (E), total GIP-AUC (F), C-peptide-AUC/glucose-AUC (G), and glucagon-AUC (H). Data represent means $\pm$ SEM.

betes duration according to the following formula: fasting C-peptide reactivity (ng/mL) = $2.151 - 0.021 \times$ diabetes duration (years) [26]. The present patients had diabetes durations of approximately 15 to 20 years. Although the formula predicts C-peptide levels of 1.73 to 1.84 for patients with such diabetes durations, their fasting C-peptide levels were in the approximate range of 1.1 to 1.3. Thus, in our patients, β -cell deterioration may have been more severe than in most type 2 diabetes patients with similar disease durations, possibly because of their poor diabetes control histories. Nonetheless, sitagliptin and metformin reduced HbA1c and glycated albumin levels in the study participants. However, these two drugs appear to differ in their effects on islet function.

In the present study, after 12-week sitagliptin treatment, we demonstrated that the DPP-4 inhibitor was effective for preventing incretin degradation, augmenting blood concentrations of active forms of GLP-1 and GIP, although total GLP-1 and total GIP secretion in response to a meal challenge were decreased. The reduced incretin secretion from L and K cells were previously observed in DPP-4 inhibitor-treated dogs [27], healthy non-Asians [28] and type 2 diabetes Caucasians [20]. Thus, possible feedback regulation of incretin secretion could also be operating in Japanese patients with type 2 diabetes treated on insulin monotherapy.

Our data demonstrated that enhanced incretin action by the DPP-4 inhibitor led to improved glucose responsiveness of β -cells and suppressed glucagon secretion from α -cells in insulin-treated patients with relatively long durations of diabetes. It was recently reported that GIP infusion antagonizes the glucagonostatic effects of GLP-1 in hyperglycemic patients with type 2 diabetes [8]. Although active GIP levels were increased by sitagliptin in the present subjects, glucagon levels were reduced, indicating that the glucagonostatic effect of increased GLP-1 was superior to the glucagonotropic effect of enhanced GIP activities.

Recently, an observational study by Nakagami *et al.* [29] and a randomized crossover study by Farngren *et al.* [30] showed DPP-4 inhibitors to reduce glucagon secretion after meal challenge in insulin-treated patients with or without oral antidiabetic drugs. The latter study also showed that vildagliptin, another DPP-4 inhibitor, did not compromise glucagon secretion during hypoglycemia, highlighting its ideal effects on glucagon dynamics for insulin-treated patients under threat of hypoglycemia. In these studies, 40% [29] and 65% [30] subjects

were also treated with metformin. Since metformin has been suggested to augment the effects of DPP-4 inhibitors by increasing production of GLP-1 [17], whether glucagon suppressing effects were caused by the DPP-4 inhibitors alone has yet to be clarified. Studying only patients with insulin monotherapy, our data showed that glucagon secretion after 12-week sitagliptin treatment was lower than that before treatment, providing strong evidence for glucagon suppressing effects of sitagliptin in insulin-treated patients.

In addition, although the observational study have provided important information of real world medicine, it lacked a control group, making the causal relationship between improved glycemic control and reduced glucagon levels unclear, since islet function is known to be modulated by glycemic control status [31]. Plasma DPP-4 activity [32] and GIP secretion [6] are also reportedly associated with HbA1c levels, likely affecting effects of DPP-4 inhibitors. Therefore, it is important to evaluate effects of DPP-4 inhibitors with appropriate controls. We observed that addition of sitagliptin or metformin resulted in similar glycemic improvements, and found that decreases in glucagon AUC by sitagliptin was significantly different from changes by metformin. Although we compared glucagon responses to MTT when sitagliptin and metformin groups showed similar HbA1c levels, glycemic levels in response to meal challenges were varied. Since islet hormone and incretin levels could be affected by glycemic levels, comparison between sitagliptin and metformin effects should be cautiously interpreted. The ideal would be to make the comparison with meal-induced glycemic levels being equally elevated. Future studies employing the clamp technique are anticipated to allow such precise comparison to be made.

Vildagliptin reduced glucagon secretion [33], but sitagliptin did not [34] in C-peptide negative type 1 diabetes patients. Although β -cell and δ -cell mediated paracrine suppressions have been proposed as the mechanism by which incretins suppress glucagon secretion, the details have yet to be established [4]. Future studies should focus on the amounts of residual β -cells and/or δ -cells needed for DPP-4 inhibitors to suppress glucagon secretion.

Another issue regarding glucagon concentrations worthy of mention is the problem of glucagon assays. As recently reported, there are as yet no commercially available glucagon immunoassay systems with ideal accuracy [35]. We used a Linco (Millipore) RIA,

which has been described as the best, though imperfect, assay currently available [35]. However, even the Linco RIA reportedly yields higher baseline measurements. The reason for this is currently unknown. If the basal values were actually smaller, the changes after sitagliptin treatment might in actuality have been even larger in the present subjects.

In metformin-treated patients, HbA1c levels were reduced despite glucose excursion during MTT being unaltered after 12 weeks. Thus, metformin must have reduced blood glucose levels early in the morning or between meals. Although active GLP-1 levels tended to increase 15 min after the meal load, integrated responses were not changed in subjects treated with metformin. This is in accordance with reports by Solis-Herrera *et al.* [19] and Vardarli *et al.* [20], but not those by Migoya *et al.* [17] and Thondam *et al.* [18]. The reason for this discrepancy is currently unknown. Differences in race, age and diabetes stages may influence metformin effects on GLP-1 levels.

We found that C-peptide responses during MTT increased after metformin treatment. This might be related to insulin doses before breakfast (thus before MTT) having been reduced in four of the 10 patients because of hypoglycemic events. Similar tendencies for rising C-peptide levels, though not statistically significant, have been reported after metformin addition to insulin-treatment regimens in two randomized trials [36, 37]. The primary hypoglycemic action of metformin is attributed to its insulin sensitizing effects in the liver and peripheral tissues [38]. However, a report suggested that metformin augmented glucose-induced insulin secretion *in vivo* [39]. In addition, the combination of metformin and sitagliptin was reported to elicit greater insulin secretion in response to a meal challenge than sitagliptin alone, suggesting that metformin stimulated insulin secretion under some circumstances [19]. It is also possible that improved glycemic control by metformin *per se* ameliorated β -cell dysfunction, for example, by re-sensitizing β -cells to GIP [6]. Effects of metformin on β -cells *in vitro* are controversial [40, 41]. Future studies are needed to analyze metformin effects on β -cell function *in vivo* in various stages of islet deterioration.

It is perhaps noteworthy that metformin treatment tended to increase fasting glucagon levels, since Vardarli *et al.* recently reported similar findings in patients treated with metformin [20]. Fasting glucagon elevation may enhance β -cell competence for insulin

secretion by raising cyclic adenosine mono-phosphate levels in these cells. In addition, when we evaluated incremental AUC (above values at time zero) instead of total AUC (above zero), we found the value to be 30% less after as compared to before metformin treatment ($P = 0.047$). This may be due to increased β -cell responsiveness and thus to increased paracrine suppression in α -cells.

The major limitation of our study is the rather small number of participants. We recruited only patients on insulin monotherapy, because possible drug interactions could cause variability in the effects of sitagliptin and/or metformin. Alpha-glucosidase inhibitors, sulfonylureas and thiazolidinediones could augment or mask effects of sitagliptin and metformin by altering incretin levels, glucagon secretion and/or DPP-4 activities. Although the data, such as the effects of sitagliptin treatment on glucagon levels, were uniform and showed statistically significant differences, increasing the number of patients enrolled would have increased the statistical power for evaluating other parameters, such as the incidence of hypoglycemia. Another limitation is that the study subjects treated with insulin monotherapy had low C-peptide levels, which may not represent the typical type 2 diabetic patients. Furthermore, we did not examine patients treated with combination of insulin and oral anti-diabetes agents. Thus, studies in patients on insulin therapy with relatively higher C-peptide values and/or treated with anti-diabetes agents in combination are needed for applying our results to general population of insulin-treated type 2 diabetes mellitus. It should also be noted that since MTT were conducted after ingestion of sitagliptin or metformin, it was unclear whether different effects of sitagliptin and metformin on islet hormone secretion were caused by single or chronic administration of each drug. To analyze chronic effects, it would be better to conduct MTT without drug ingestion as in the studies by Solis-Herrera *et al.* [19] and Vardarli *et al.* [20], while MTT with prior ingestion as in the present study and a study by Ahren *et al.* [9] would more closely reflect real-world medicine.

In conclusion, our data is the first to demonstrate that sitagliptin suppress glucagon secretion in response to a meal challenge and to provide data regarding metformin effects on incretin levels and islet hormone secretion in Japanese type 2 diabetic patients on insulin monotherapy. A glucagon suppressing effect of sitagliptin may contribute to improvement of blood glu-

cose control in patients inadequately controlled with insulin therapy.

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Competing Interests

None of the authors have any potential conflicts of interest associated with this research.

References

- Babbio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132: 2131-2157.
- Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, et al. (1993) Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest* 91: 301-307.
- Meier JJ, Gallwitz B, Siepmann N, Holst JJ, Deacon CF, et al. (2003) Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycemia. *Diabetologia* 46: 798-801.
- Holst JJ, Christensen M, Lund A, de Heer J, Svendsen B, et al. (2011) Regulation of glucagon secretion by incretins. *Diabetes Obes Metab* 13 suppl 1: 89-94.
- Nauck MA, Vardarli I, Deacon CF, Holst JJ, Meier JJ (2011) Secretion of glucagon-like peptide 1 (GLP-1) in type 2 diabetes: what is up, what is down? *Diabetologia* 54: 10-18.
- Calanna S, Christensen M, Holst JJ, LaFerrere B, Gluud LL, et al. (2013) Secretion of glucose-dependent insulinotropic peptide in patients with type 2 diabetes: systemic review and meta-analysis of clinical trials. *Diabetes Care* 36: 3346-3352.
- Lund A, Visboll T, Bagge JI, Holst JJ, Knop FK (2011) The separate and combined impact of the intestinal hormones, GIP, GLP-1, and GLP-2, on glucagon secretion in type 2 diabetes. *Am J Physiol Endocrinol Metab* 300: E1038-E1046.
- Mentis N, Vardarli I, Kothe LD, Holst JJ, Deacon CF, et al. (2011) GIP does not potentiate the antidiabetic effects of GLP-1 in hyperglycemic patients with type 2 diabetes. *Diabetes* 60: 1270-1276.
- Ahren B, Schweizer A, Dejager S, Dunning BE, Nilsson PM, et al. (2009) Vildagliptin enhances islet responsiveness to both hyper- and hypoglycemia in patients with type 2 diabetes. *J Clin Endocrinol Metab* 94: 1236-1243.
- Henry RR, Smith SR, Schwartz SL, Mudaliar SR, Deacon CF, et al. (2011) Effects of saxagliptin on β -cell stimulation and insulin secretion in patients with type 2 diabetes. *Diabetes Obes Metab* 13: 850-858.
- Muscelli E, Casolaro A, Gastaldelli A, Mari A, Seghieri G, et al. (2012) Mechanisms for the antihyperglycemic effect of sitagliptin in patients with type 2 diabetes. *J Clin Endocrinol Metab* 97: 2818-2826.
- Rosenstock J, Rendell MS, Gross JL, Fleck PR, Wilson CA, et al. (2009) Alogliptin added to insulin therapy in patients with type 2 diabetes reduces HbA1c without causing weight gain or increased hypoglycemia. *Diabetes Obes Metab* 11: 1145-1152.
- Shimoda S, Iwashita S, Ichimori S, Matsuo Y, Goto R, et al. (2013) Efficacy and safety of sitagliptin as add-on therapy on glycemic control and blood glucose fluctuation in Japanese type 2 diabetes subjects ongoing with multiple daily insulin injections therapy. *Endocr J* 60: 1207-1214.
- Katsuno T, Ikeda H, Ida K, Miyagawa J, Namba M (2013) Add-on therapy with the DPP-4 inhibitor sitagliptin improves glycemic control in insulin-treated Japanese patients with type 2 diabetes mellitus. *Endocr J* 60: 733-742.
- Viltsbøll T, Rosenstock J, Yki-Jarvinen H, Cefalu WT, Chen Y, et al. (2010) Efficacy and safety of sitagliptin when added to insulin therapy in patients with type 2 diabetes. *Diabetes Obes Metab* 12: 167-177.
- Kothny W, Foley J, Kozlovski P, Shao Q, Gallwitz B, et al. (2013) Improved glycaemic control with vildagliptin added to insulin, with or without metformin, in patients with type 2 diabetes mellitus. *Diabetes Obes Metab* 15: 252-257.
- Migoya EM, Bergeron R, Miller JL, Snyder RNS, Tanen M, et al. (2010) Dipeptidyl peptidase-4 inhibitors administered in combination with metformin result in an additive increase in the plasma concentration of active GLP-1. *Clin Pharmacol Ther* 88: 801-808.
- Thondam SK, Cross A, Cuthbertson DJ, Wilding JP, Daousi C (2012) Effects of chronic treatment with metformin on dipeptidyl-peptidase-4 activity, glucagon-like peptide 1 and ghrelin in obese patients with type 2 diabetes mellitus. *Diabetic Med* 29: e205-e210.
- Solis-Herrera C, Triplitt C, Garduno-Garcia JJ, Adams J, DeFronzo RA, et al. (2013) Mechanisms of glucose lowering of dipeptidyl peptidase-4 inhibitor sitagliptin when used alone or with metformin in type 2 diabetes: a double-tracer study. *Diabetes Care* 36: 2756-2762.

20. Vardarli I, Arndt E, Deacon CF, Holst JJ, Nauck MA (2014) Effects of sitagliptin and metformin treatment on incretin hormone and insulin secretory responses to oral and "isoglycemic" intravenous glucose. *Diabetes* 63: 663-674.
21. Hemmingsen B, Christensen LL, Wetterslev J, Vaag A, Gluud C, et al. (2012) Comparison of metformin and insulin versus insulin alone for type 2 diabetes: systemic review of randomized clinical trials with meta-analysis and trial sequential analyses. *BMJ* 344: e1771.
22. Yoshino G, Tominaga M, Hirano T, Shiba T, Kashiwagi A, et al. (2006) The test meal A: a pilot model for the international standard of test meal for assessment of both postprandial hyperglycemia and hyperlipidemia. *J Jpn Diabetes Soc* 49: 361-371 (In Japanese).
23. Yabe D, Watanabe K, Sugawara K, Kuwata H, Kitamoto Y, et al. (2012) Comparison of incretin immunoassays with or without plasma extraction: Incretin secretion in Japanese patients with type 2 diabetes. *J Diabetes Investig* 3: 70-79.
24. Masuda K, Aoki K, Terauchi Y (2012) Comparison of plasma active glucagon-like peptide-1 (GLP-1) levels assayed with or without plasma extraction in non-diabetic men. *Endocr J* 59: 435-438.
25. Chan JC, Malik V, Jia W, Kadowaki T, Yajnik CS, et al. (2009) Diabetes in Asia: epidemiology, risk factors, and pathophysiology. *JAMA* 301: 2129-2140.
26. Funakoshi S, Fujimoto S, Hamasaki A, Fujiwara H, Fujita Y, et al. (2008) Analysis of factors influencing pancreatic beta-cell function in Japanese patients with type 2 diabetes: association with body mass index and duration of diabetic exposure. *Diabetes Res Clin Pract* 82: 353-358.
27. Deacon CF, Wamberg S, Bie P, Hughes TE, Holst JJ (2002) Preservation of active incretin hormones by inhibition of dipeptidylpeptidase IV suppresses meal-induced incretin secretion in dogs. *J Endocrinol* 172: 355-362.
28. El-Ouaghli A, Rehling E, Holst JJ, Schweizer, Foley J, et al. (2007) The dipeptidyl peptidase 4 inhibitor vildagliptin does not accentuate glibenclamide-induced hypoglycemia but reduces glucose-induced glucagon-like peptide 1 and gastric inhibitory polypeptide secretion. *J Clin Endocrinol Metab* 92: 4165-4171.
29. Nakagami T, Ide R, Iwasaki N, Ogata M, Oya J, et al. (2014) Evaluation of add-on therapy of sitagliptin in Japanese patients with type 2 diabetes under insulin therapy. *Diabetol Int* 5: 187-197.
30. Farngren J, Persson M, Schweizer A, Foley JE, Ahren B (2014) Glucagon dynamics during hypoglycemia and food-re-challenge following treatment with vildagliptin in insulin-treated patients with type 2 diabetes. *Diabetes Obes Metab* 16: 812-818.
31. Kosaka K, Kuzuya T, Akanuma Y, Hagura R (1980) Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. *Diabetologia* 18: 23-28.
32. Ryskjaer JI, Deacon CF, Carr RD, Krarup T, Madsbad S, et al. (2006) Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbA1c levels, but is not acutely affected by food intake. *Eur J Endocrinol* 155: 485-493.
33. Farngren J, Persson M, Schweizer A, Foley JE, Ahren B (2012) Vildagliptin reduces glucagon during hyperglycemia and sustains glucagon counterregulation during hypoglycemia in type 1 diabetes. *J Clin Endocrinol Metab* 97: 3799-3806.
34. Garg SK, Moser EG, Bode BW, Klaff LJ, Hiatt WR, et al. (2013) Effect of sitagliptin on postprandial glucagon and GLP-1 levels in patients with type 1 diabetes: investigator-initiated, double-blind, randomized, placebo-controlled trial. *Endocr Pract* 19: 19-28.
35. Bak MJ, Albrechtsen NW, Pedersen J, Hartmann B, Christensen M, et al. (2014) Specificity and sensitivity of commercially available assays for glucagon and oxyntomodulin measurement in human. *Eur J Endocrinol* 170: 529-538.
36. Aviles-Santa L, Sinding J, Raskin P (1999) Effects of metformin in patients with poorly controlled insulin-treated type 2 diabetes mellitus. *Ann Intern Med* 131: 182-188.
37. Yu JG, Kruszynska YT, Nulford MI, Olefsky JM (1999) A comparison of troglitazone and metformin on insulin requirements in euglycemic intensively insulin-treated type 2 diabetes patients. *Diabetes* 48: 2414-2421.
38. Andújar-Plata P, Pi-Sunyer X, Laferrère B (2012) Metformin effects revisited. *Diabetes Res Clin Pract* 95: 1-9.
39. Marchetti P, Scharp DW, Giannarelli R, Benzi L, Cicchetti P, et al. (1996) Metformin potentiates glucose-stimulated insulin secretion. *Diabetes Care* 19: 781-782.
40. Leclerc I, Woltersdorf WW, da Silva Xavier G, Rowe RL, Cross SE, et al. (2004) Metformin, but not leptin, regulates AMP-activated protein kinase in pancreatic islets: impact on glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab* 286: E1023-E1031.
41. Marchetti P, Guerra SD, Marselli L, Lupi R, Masini M, et al. (2004) Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab* 89: 5535-5541.