博士論文

Contribution of Pancreatic Alpha Cell Function to Insulin Sensitivity and Glycemic Variability in Type 1 Diabetes

（ヒト１型糖尿病における膵α細胞予備能とインスリン抵抗性・血糖変動との関連性）

高橋　信行

Contribution of Pancreatic Alpha Cell Function to Insulin Sensitivity and Glycemic Variability in Patients with Type 1 Diabetes

Affiliation:

Department of Molecular Diabetology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Graduate Educator: Toshimasa Yamauchi, MD, PhD

Author: Nobuyuki Takahashi, MD

Abstract

Introduction: In the context of type 1 diabetes, the pathophysiologic roles of glucagon in glucose metabolism remain unclear. Novel glucagon enzyme-linked immunosorbent assays (ELISA) have been developed. This study evaluated the contribution of pancreatic alpha cell function to dawn phenomenon, insulin sensitivity, hepatic glucose uptake (HGU), and glycemic variability in type 1 diabetes.

Methods: In 40 patients with type 1 diabetes, the area under the curve of glucagon response to arginine stimulation was measured using conventional radioimmunoassay (AUC_{glcRIA}) and ELISA ($AUC_{glcELISA}$). The ratio of the insulin dose to maintain euglycemia between 04:00 and 08:00 or between 00:00 and 04:00 was measured as the dawn index. The glucose infusion rate (GIR) and HGU were measured using a hyperinsulinemic euglycemic clamp and clamp oral glucose loading tests. Glycemic variability in 96 h was measured by continuous glucose monitoring (CGM).

Results: Median dawn index (= 1.7 [1.0 – 2.8]) was not correlated with AUC_{glcRIA} (R^2 = 0.03, P = 0.39) or $AUC_{glcELISA}$ (R^2 = 0.04, P = 0.32). Median GIR (= 7.3 [6.4–9.2] mg/kg/min) was significantly correlated with AUC_{glcRIA} (R^2 = 0.20, P = 0.02) and $AUC_{glcELISA}$ (R^2 = 0.21, P = 0.02). Median HGU (= 65.3 [40.0 – 87.3]) was not correlated with AUC_{glcRIA} (R^2 = 0.07, P = 0.26) or $AUC_{glcELISA}$ (R^2 = 0.26, P = 0.79). Standard

deviation of glucose levels was significantly correlated with AUC_{glcRIA} ($R^2 = 0.11$, $P = 0.049$) but not with $AUC_{glcELISA}$ ($R^2 = 0.01$, $P = 0.75$).

Conclusions: Pancreatic alpha cell function contributed to insulin sensitivity in type 1 diabetes.

Introduction

Bihormonal hypothesis

Type 1 diabetes mellitus is characterized by insulin deficiency caused by pancreatic beta cell destruction [1]. The glucagon, which is secreted by pancreatic alpha cells, increases blood glucose levels through several mechanisms, such as activation of glycogenolysis in the liver and peripheral soft tissue. More than 30 years ago, Unger and Orci proposed that glucagon, in the context of absolute insulin deficiency, contributes to hyper- or hypoglycemia in type 1 diabetes: the so-called “byhormonal hypothesis” [2]. Indeed, impaired suppression of glucagon after oral ingestion of glucose has been demonstrated in patients with type 1 diabetes [3], whereas glucagon response to hypoglycemia is also impaired in patients with type 1 diabetes, as compared to healthy individuals [4]. Thus, in addition to insulin deficiency, defects in glucagon physiology may be involved in aggravating hyper- or hypoglycemia in patients with type 1 diabetes. However, the mechanisms underlying the aberrant glucagon response of pancreatic alpha cells in type 1 diabetes remains ill-defined. Postprandial hypersecretion of glucagon has been attributed to the relative lack of intra-islet insulin or the insensitivity of the pancreatic alpha cells to the direct inhibitory effects of glucose [5, 6]. In addition, recent studies have also implicated certain incretin hormones, such as glucagon-like peptide-1 (GLP-1), as

potentiating glucagonotropic mediators of postprandial hyperglucagonemia in patients with type 1 diabetes [7].

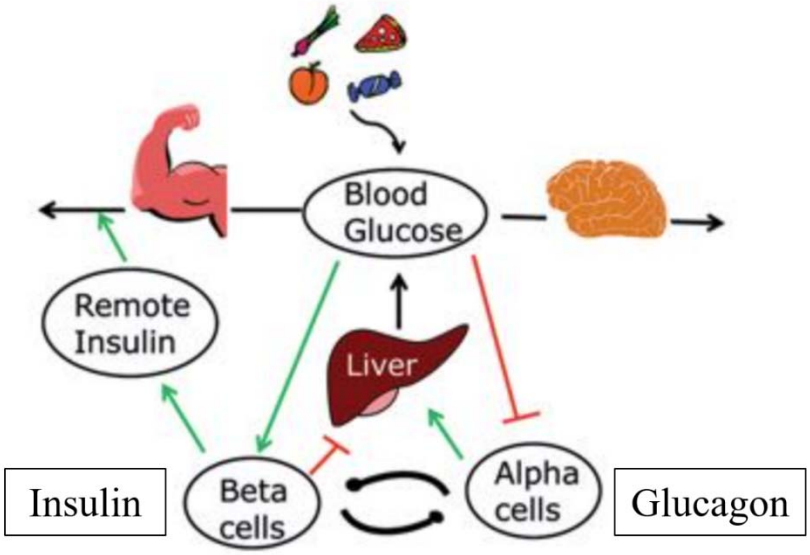

Figure S1. The pathophysiological relationship among insulin, glucagon and several organs in glucose metabolism

Characteristic glucose metabolism mechanisms in type 1 diabetes

Notably, patients with type 1 diabetes typically exhibit glucose metabolism mechanisms that are different from those of healthy individuals, including reduced insulin sensitivity, acutely increased blood glucose levels from midnight to early morning (called the “dawn phenomenon”), impaired hepatic glucose uptake (HGU), and unstable glycemic variability.

Reduced insulin sensitivity in patients with type 1 diabetes

Reduced insulin sensitivity typically characterizes patients with diabetes or pre-diabetic condition and is the hallmark of the metabolic syndrome [8]. Even if the primary metabolic defect of type 1 diabetes is considered to be insulin deficiency, a number of studies suggested that a certain degree of reduced insulin sensitivity is also present in patients with type 1 diabetes. Adolescent and adult patients with type 1 diabetes show reduced insulin sensitivity, even when compared to non-diabetic counterparts of similar adiposity, body fat distribution, serum triglycerides, high-density lipoprotein cholesterol, and level of habitual physical activity, all of which are factors that contribute to insulin sensitivity [9]. The list of risk factors for reduced insulin sensitivity in patients with type 1 diabetes also includes prolonged peripheral exposure to supraphysiologic levels of exogenous insulin, genetic and environmental factors, and impaired delivery of insulin to the portal circulation [10-12].

The role of insulin sensitivity in the development and progression of macro- (i.e., myocardial infarction, stroke, and peripheral arterial disease) and microvascular (diabetic retinopathy, nephropathy, and neuropathy) complications in patients with type 1 diabetes has been increasingly recognized. In the Pittsburg Epidemiology of Diabetes Complications cohort study which included 603 patients with type 1 diabetes and

followed up cardiovascular events over 10 years, independent predictors of cardiovascular events were disease duration, presence of nephropathy, non-HDL-cholesterol level, white blood cell count, and glucose infusion rate (GIR) evaluated by hyperinsulinemic euglycemic clamp test, which is the method used to accurately evaluate insulin sensitivity [13]. In an independent examination of Diabetes Complications Control Trial/Epidemiology of Diabetes Interventions and Complications data (a large prospective randomized clinical trial, which proved that strict glycemic control by insulin intensive therapy could improve diabetic complications in patients with type 1 diabetes), Kilpatrick *et al*. retrospectively identified GIR as a predictor of cardiovascular events as well [14]. In another prospective cohort study, Bjornstad et al. reported that baseline insulin sensitivity was associated with the development of diabetic nephropathy in patients with type 1 diabetes [15]. It is therefore essential to better understand the pathophysiological factors which contribute to reduced insulin sensitivity.

Dawn phenomenon in patients with type 1 diabetes

The dawn phenomenon, first reported by Schmidt *et al*. [16], refers to an acute increase of blood glucose levels between 04:00 and 08:00, which is typically observed in patients with type 1 diabetes. The increase in glucose level can exceed 200 mg/dl in the morning;

however, the magnitude of increased insulin requirement varies considerably among patients and even from day to day within a patient. Previous studies suggest that in patients with type 1 diabetes, insulin requirement during this period increased as much as six folds, with an average increase of up to 180% [17]. Several hypotheses accounting for the dawn phenomenon have been put forward. Initially, circadian variation in counterregulatory hormones such as growth hormone (GH) or insulin-like growth factor-1 (IGF-1) seems to contribute to the dawn phenomenon. Campbell *et al*. inhibited the rise of the growth hormone with somatostatin and concomitantly diminished the dawn rise in blood glucose; furthermore, when GH and somatostatin were administered together, blood glucose rose again [18]. Davidson *et al*. have also demonstrated that anticholinergic blockade of nocturnal GH attenuated morning glucose rise [19]. Recent studies indicate that the dawn phenomenon could affect overall glycemic control. Monnier *et al*. reported an increase in blood glucose level of 13-20 mg/dl from nocturnal nadir to pre-breakfast values and the blood glucose elevation related with post-breakfast and the highest value of the day. More importantly, the blood glucose level elevation also impacted the glycated hemoglobin (HbA1c) level [20].

Impaired hepatic glucose uptake in patients with type 1 diabetes

Although skeletal muscle is an essential site for insulin sensitivity, the liver also plays a critical role in glucose metabolism. The liver can both export glucose for use or by other organ tissues by glycogenosis and extract glucose to reduce glycemia by gluconeogenesis. Failure of the liver to make the transition from net output to the uptake of glucose in the postprandial state is a significant contributor to the development of impaired glucose tolerance and hyperglycemia [21, 22]. In a previous report of patients with type 1 diabetes using ^{13}C nuclear magnetic resonance spectroscopy, the measured HGU was significantly decreased by only 30%, as compared with healthy individuals [23]. Authors linked impaired HGU levels with higher rates of hepatic glycogen cycling, due to increased activation of glycogen synthase and phosphorylase, or decreased activation of hepatic glucokinase [24, 25].

Unstable glycemic variability in patients with type 1 diabetes

In previous large prospective cohort studies, the importance of glycemic control with intensive insulin therapy has been observed for preventing macro- or microvascular diabetic complications in patients with type 1 diabetes [26, 27]. However, glycemic variability in patients with type 1 diabetes is typically unstable, and they have a high risk of severe hyperglycemia or hypoglycemia. Unstable glucose control in patients with type

1 diabetes mainly seems to be associated with irreversible loss of insulin secretion and thus difficulty with adjusting insulin treatment [28]. Unstable glycemic variability can contribute to diabetes complications [29], decreasing their quality of life [30], and can also cause nocturnal hypoglycemia, which can contribute to fatal conditions called “the dead-in bead syndrome” [31].

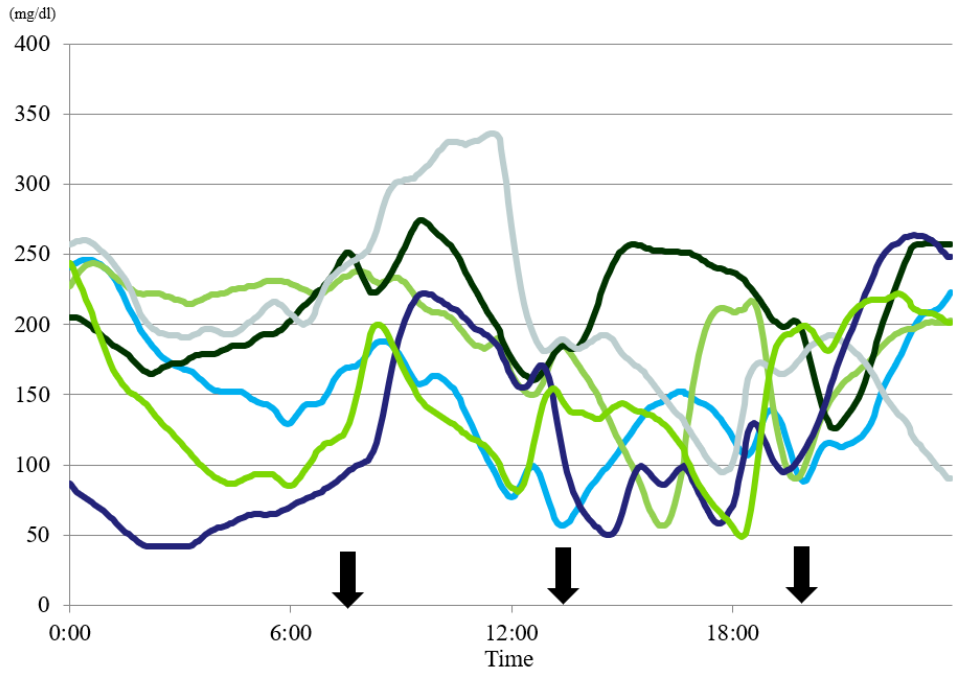

Figure S2. Unstable glycemic variability in a patient with type 1 diabetes. The patient is 49 years old female with type 1 diabetes diagnosed 10 years ago and her insulin secretion was diminished based on arginine stimulation test (Her serum C-peptide was not responded by arginine stimulation). The patient manages blood glucose levels through intensive insulin therapy that includes insulin lispro injections (rapid-acting insulin), of 5–10 units (she adjusts based on the carbons she takes) before each meal, and insulin degludec (long-acting insulin) administration for 15 units at bedtime. The glucose levels were continuously measured by continuous glucose monitoring (CGM, ipro2; Medtronic Minimed, CA, USA).

Glucagon assay

Despite the extensive studies mentioned above, the role of a glucagon as a pathophysiologic factor of glucose metabolism in patients with type 1 diabetes remains unclear. One of the possible reasons for this is the inaccuracy of the conventional glucagon assay used in previous studies. Proglucagon is converted into glucagon by proglucagon convertase [32]. Along with this process, other proglucagon fragments (e.g., oxyntomodulin, glicentin, and GLP-1) are also produced. Measurements of glucagon with the radioimmunoassay (RIA) kits, the conventional glucagon assay, require polyclonal antibodies against the glucagon C-terminal region, and these antibodies cross-react with other proglucagon fragments that also contain the C-terminal region. In contrast, a novel double-sandwich enzyme-linked immunosorbent assay (ELISA) kit uses monoclonal antibodies against both the C- and N-terminal regions of glucagon and measure glucagon concentrations with much lower crossreactivity against proglucagon fragments other than glucagon (1–29) [33]. In a previous study, the accuracy of glucagon levels measured by ELISA kit was confirmed with novel liquid chromatography-high resolution mass spectroscopy [34]. Furthermore, the authors also observed that the trend of glucagon levels measured by ELISA kit differed from that measured by RIA kit during meal

tolerance test; the former returned slightly high results whereas the latter produced significantly lower levels. Further evaluation for pancreatic alpha cell function with these different kits is warranted.

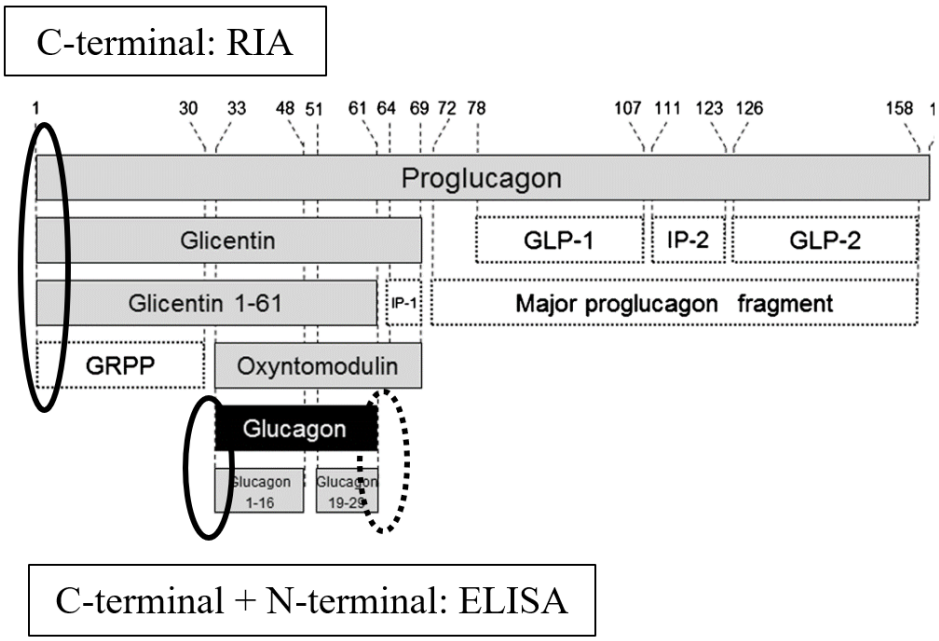

Figure S3. Difference between RIA and ELISA to evaluate Glucagon. Circles with solid lines and dashed line show C-terminal and N-terminal, respectively; RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbent assay; GLP: glucagon-like polypeptide; IP: intervening peptide; GRPP: glicentin-related pancreatic polypeptide.

Aim of the study

Accordingly, in this study, I aimed to determine the contribution of pancreatic alpha cell function evaluated with RIA or ELISA kits to insulin sensitivity, HGU, and glycemic variability, including the dawn phenomenon, in patients with type 1 diabetes.

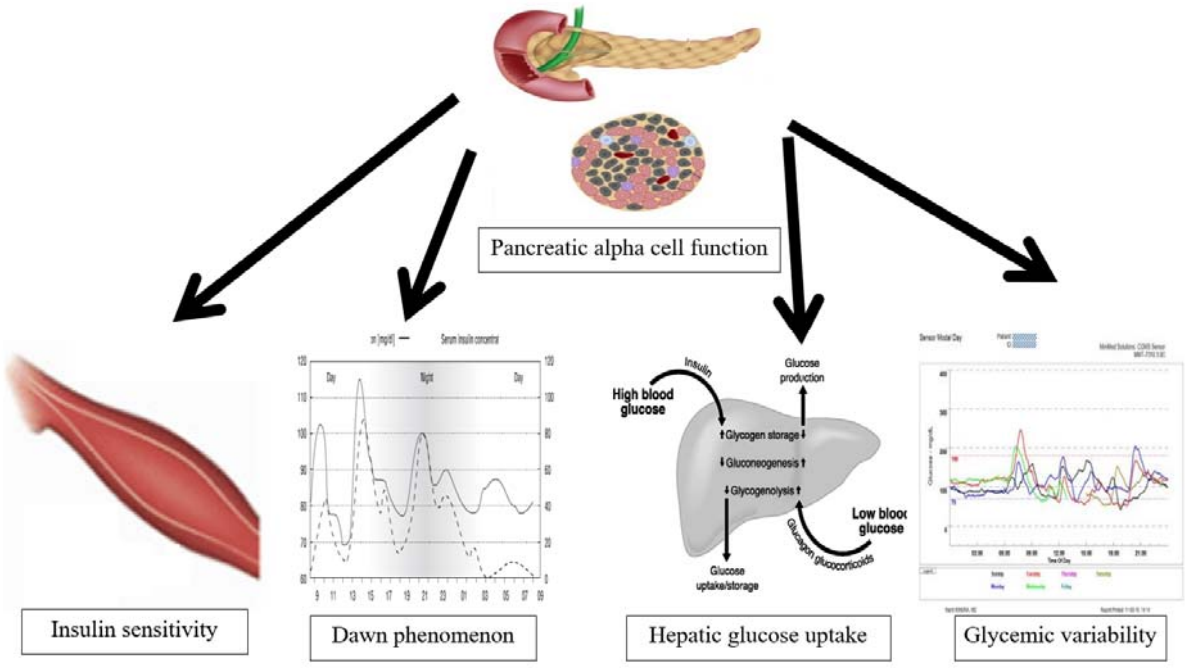

Figure S4. The aim of this study.

Materials and Methods

Study design and patients

This cross-sectional study was carried out at the National Center for Global Health and Medicine in Tokyo, Japan. I examined patients diagnosed with type 1 diabetes who were admitted to my hospital and met inclusion criteria and unmet exclusion criteria. Inclusion criteria were as follows: patients who were previously diagnosed with type 1 diabetes according to World Health Organization criteria [35] and were at least 20 years of age. Exclusion criteria were as follows: current treatment with steroid hormones or

immunosuppressants; pregnant or breastfeeding; estimated glomerular filtration rate (GFR) of less than 45 mL/min/1.73 m^2; current infection; and refusal to participate in the study. The estimated GFR was calculated using the following formula [36]: estimated GFR (mL/min/1.73 m^2) = 194 × (serum creatinine level, mg/dL)$^{-1.094}$ × (age, years)$^{-0.287}$ (× 0.739 if the patient was female). According to a previous report, renal insufficiency could affect the glucagon levels as it can increase blood glucagon levels by decreasing urinary excretion of glucagon [37]; hence, patients with renal insufficiency were excluded. Baseline characteristic information was collected from patient medical records. Measurements as baseline characteristics were as follows: age, sex, body mass index (BMI, calculated as weight in kilograms divided by height in meters squared), diabetes duration, glycated hemoglobin (HbA_{1c}), fasting levels of serum C-peptide, estimated GFR, insulin treatment regimen (multiple daily injection or continuous subcutaneous insulin infusion), total daily insulin dose, and basal/bolus ratio. All patients provided informed and written consent. This study conformed to the provisions of the Declaration of Helsinki and was approved by the Institutional Review Board of the National Center for Global Health and Medicine (No. 2020).

Arginine stimulation test

On admission, each patient underwent arginine stimulation tests to evaluate their pancreatic alpha cell function. To exclude the effects of exogenous insulin, the typical basal insulin regimen within 24 h before the arginine stimulation test was replaced with a continuous intravenous insulin injection and was stopped 1 h before the arginine stimulation test if the patient was treated with multiple daily injections. If the patient was treated with continuous subcutaneous insulin infusion, treatment with an insulin pump was continued as usual and stopped 1 h before the arginine stimulation test. Patients were asked to rest for 30 min after overnight fasting, and 30 g arginine was intravenously administrated as 10% L-arginine hydrochloride over 30 min. Blood samples were collected before and 15, 30, 60, 90, and 120 min after arginine loading. The levels of plasma glucose, serum C-peptide, and plasma glucagon were measured at each time point. The levels of plasma glucose were measured using a glucose oxidase-immobilized membrane-H_2O_2 electrode (glucose analyzer GA-1172; Arkray, Kyoto, Japan; the intra- and interassay coefficients of variation were less than 2.0%). The levels of serum C-peptide were measured by electrochemiluminescence immunoassays (Roche Diagnostics, Mannheim, Germany; the intra- and interassay coefficients of variation were 1.9% and 2.3%, respectively). The levels of plasma glucagon were measured by RIA (Sceti Medical Labo, Tokyo, Japan; the intra- and interassay coefficients of variation were less than 20%

and less than 15%, respectively, the measurement range was from 16.3 to 522 pg/ml) and sandwich ELISA (Mercodia AB, Sweden; the intra- and interassay coefficients of variation were 7.3–9.4% and 7.5–8.5%, respectively, measurement range was from 5 to 414 pg/ml). The area under the concentration-time curve (AUC) of plasma glucagon between 0 and 120 min was calculated using the trapezoidal rule. The AUC of plasma glucagon measured by RIA kits was defined as $AUC_{glcRIA,}$ and that measured by ELISA kits was defined as $AUC_{glcELISA}$. A peak glucagon level measured by RIA during arginine stimulation tests of equal to or more than 300 pg/mL was evaluated as glucagon hyperreactivity, whereas that of less than 300 pg/mL was evaluated as glucagon hyporeactivity, as previously reported [38].

Evaluation of changes in insulin requirements between night and morning as the "dawn phenomenon."

After the arginine stimulation test, I evaluated changes in insulin requirements between night and morning as the "dawn phenomenon." Continuous intravenous or subcutaneous insulin infusion resumed after arginine stimulation tests and stopped at 19:00. At 20:00, two cannulas were placed in a forearm vein (for infusion of glucose and insulin) and in a heated contralateral forearm vein (for arterialized venous blood sampling) and then

connected to an artificial pancreas (STG55; Nikkiso Co., Shizuoka, Japan). The artificial pancreas automatically primed insulin (Humulin R [Eli Lily and Company, Indianapolis, IN, USA] 250 U in 500 mL saline) in accordance with an algorism to maintain blood glucose levels within the range of 80 to 110 mg/dL throughout the test. Blood was continuously sampled, and glucose levels were measured with a glucose sensor electrode and glucose oxidase membrane every minute. The pump delivering insulin and the glucose sensor electrode each had an accuracy of ± 5% according to a previous report [39]. I evaluated changes in insulin requirements from 00:00 to 08:00 as the "dawn phenomenon" using this artificial pancreas. The ratio of the delivered insulin dose average between 04:00 and 08:00 to that between 00:00 and 04:00 was calculated as the dawn index (Figure 1).

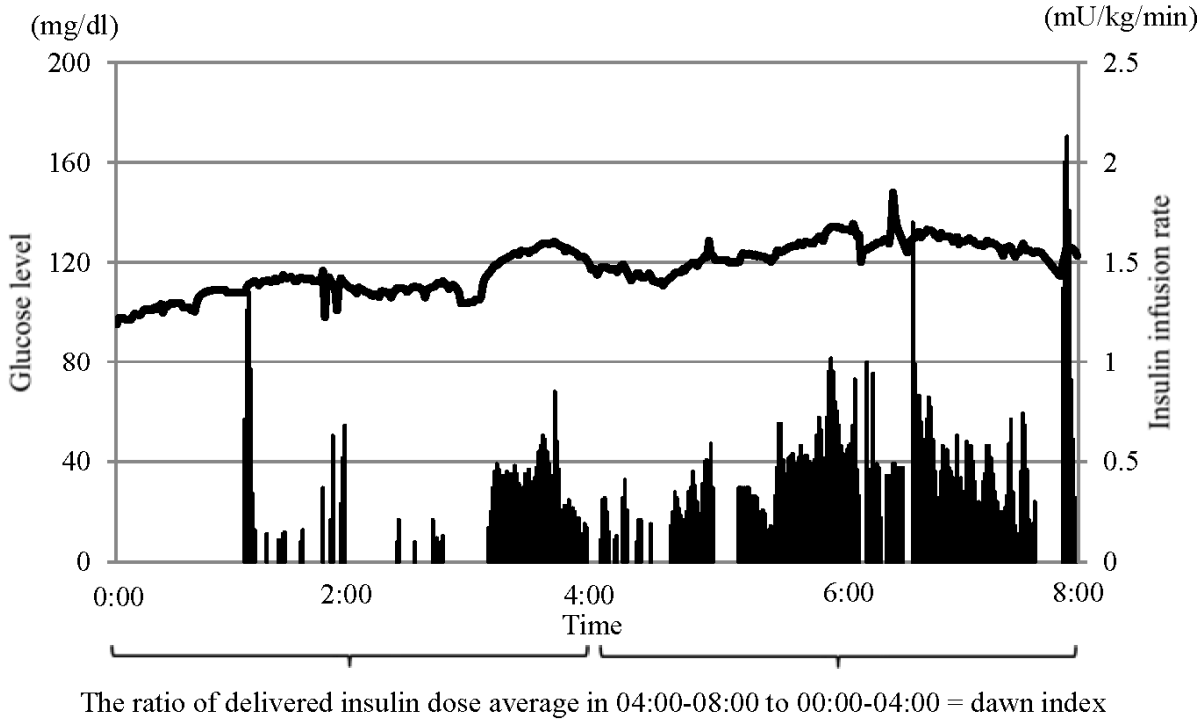

Figure 1. Schematic presentation of changes in insulin requirements between night and morning, as evaluated using an artificial pancreas. The dawn index was calculated as the ratio of the average insulin dose delivered between 04:00 and 08:00 to that between 00:00 and 04:00. Solid line: glucose level (mg/dL, left axis); bar graph: insulin infusion rate per patient weight (mU/kg/min, right axis).

To support the relationship between the dawn phenomenon and glucose-related hormones, I also measured levels of the following hormones after patients were kept at rest for 30 min after overnight fasting: GH (Elecsys immunoassay; Roche Diagnostics); IGF-1 (immunoradiometric assay; Fujirebio, Tokyo, Japan); adrenocorticotropic hormone (ACTH; Elecsys immunoassay; Roche Diagnostics); cortisol (chemiluminescent immunoassay; Siemens Medical Solutions Diagnostics, CA, USA); active GLP-1 (ELISA; IBL, Hamburg, Germany); and somatostatin (enzyme immunoassay; R&D

Systems, Minneapolis, MN, USA).

Hyperinsulinemic euglycemic clamp test

Hyperinsulinemic euglycemic clamp tests were applied to determine insulin sensitivity using the modified technique described by DeFronzo *et al*. [40]. At 8:00 after evaluating the dawn index, a primed-constant infusion of insulin was given at a rate of 2.58 mU/kg/min by the artificial pancreas to achieve a desired steady-state plasma insulin concentration (200 μU/mL). Splanchnic glucose uptake was decreased when the peripheral insulin concentration was raised to such a level [41]. Subsequently, exogenous glucose infusion was initiated to maintain blood glucose levels within the euglycemic range (95 mg/dL) throughout the study. The blood glucose level was measured every minute, and the exogenous glucose infusion rate (GIR; mg/kg/min) was adjusted by the artificial pancreas. Blood samples to measure levels of serum insulin were taken from a heated superficial hand vein 90 min after achieving steady-state. The average of GIR during the last 90 min after achieving a steady-state was calculated as an indicator of the insulin sensitivity of peripheral tissue.

Clamp oral glucose loading test

After hyperinsulinemic euglycemic clamp tests, clamp oral glucose loading tests were performed to evaluate HGU as previously described [42]. Briefly, 90 min after the blood glucose concentration monitored by the artificial pancreas reached a steady-state level, a fixed amount of glucose (0.2 g/kg) was orally administered. GIR then started to decrease because some of the ingested glucose that was not extracted by the splanchnic tissues entered the systemic circulation and reduced the GIR required to maintain euglycemia. Following an oral glucose load, in addition to the ingested glucose, recirculating glucose from the systemic circulation was presented to the liver (the HGU). The GIR required to maintain euglycemia then returned to a normal level (approximately 120 min after oral glucose administration). I calculated HGU (%) using the following formula: HGU (%) = ([oral glucose load] – [GIR decrements]) / [oral glucose load]). If the GIR decreased to zero after glucose loading, the results were excluded from the analysis. To support the relationship between HGU and glucose-related hormones, I also analyzed the correlation between HGU and fasting levels of GH, IGF-1, ACTH, cortisol, active GLP-1, and somatostatin.

Assessment of glycemic variability

Twenty-four hours after completion of tests using the artificial pancreas, each patient

underwent continuous glucose monitoring (CGM, ipro2; Medtronic Minimed, CA, USA) for 96 h. The averages of the following variables over 3 days were calculated using the CGM data: mean blood glucose level, standard deviation (SD), M-value [43], mean amplitude of glycemic excursions (MAGE) [44], hyperglycemic time, and hypoglycemic time. Hyperglycemic and hypoglycemic times were defined as the average number of minutes during which the patient's glucose levels were greater than 180 or less than 70 mg/dL in 1 day, respectively.

Statistical analysis

Mann-Whitney U tests were used to examine continuous variables, whereas Fisher's exact tests were used for two categorical variables. Pearson correlation analysis was performed to analyze the correlations among measurements. Multiple regression analysis was performed to examine the relationships between GIR during hyperinsulinemic euglycemic clamp assays as the dependent variable and the following independent variables: model 1 included age, sex, BMI, and AUC_{glcRIA}; model 2 included age, sex, BMI, and $AUC_{glcELISA}$. Results with *P* values of less than 0.05 were considered statistically significant. All analyses were performed using STATA software, version 14.2 (StataCorp, College Station, TX, USA).

Results

Demographics

In total, 40 Japanese patients with type 1 diabetes who met the inclusion criteria and unmet the exclusion criteria participated in this study. Table 1 shows the patients' characteristics. Briefly, the diabetes duration was short, the patients were not obese, and the median fasting level of serum C-peptide was less than 1.0 ng/mL, suggesting that their beta cell function was severely impaired.

Table 1. Clinical characteristics of the patients included in this study.

	N = 40
Age (years)	43 (31–56)
Female	21 (52.5%)
BMI (kg/m^2)	20.5 (19.0–21.7)
Diabetes duration (years)	2.6 (0.08–10.3)
HbA1c (%)	8.2 (7.4–10.3)
(mmol/mol)	66 (57–89)
Fasting serum C-peptide (ng/mL)	0.32 (0.00–0.94)

Estimated GFR (mL/min/1.73 m^2)	111.1 (83.3–124.1)
Insulin treatment	
MDI/CSII	32/8
Total daily insulin dose per weight (units/day/kg)	0.50 (0.33–0.75)
Basal/bolus ratio	0.42 (0.30–0.61)

Data are presented as n, n (%), or median (interquartile range). BMI: body mass index calculated by weight in kilograms divided by height in meters squared; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate calculated using the following formula [36]: estimated GFR (mL/min/1.73 m^2) = 194 × (serum creatinine level, mg/dL)$^{-1.094}$ × (age, years)$^{-0.287}$ (× 0.739 if the patient was female); MDI: multiple daily injection; CSII: continuous subcutaneous insulin infusion.

Glucagon response to arginine stimulation measured by RIA or ELISA

Figure 2A and 2B show plasma glucose, serum C-peptide, and plasma glucagon levels measured by RIA or ELISA curves in response to arginine stimulation. The levels of plasma glucose were increased in response to arginine stimulation. The response of serum C-peptide in almost all patients was abolished, although a slight response was observed in some patients (Figure 2A). Median (interquartile range) plasma glucagon levels at preloading and peak as measured by RIA and the AUC_{glcRIA} were 133.5 (117.0–151.5)

pg/mL, 413.0 (272.5–507.0) pg/mL and 3.7 (2.6–4.6) × 10^4 pg/mL·min, respectively, and those measured by ELISA were 2.5 (0–7.0) pg/mL, 32.8 (10.7–61.2) pg/mL, and 2.0 (0.8–4.5) × 10^3 pg/mL·min, respectively. Trends in the glucagon response to arginine stimulation as measured by RIA or ELISA were similar (Figure 2B). Correlations in the levels of plasma glucagon measured by RIA and ELISA at preloading and peak and those between logarithm-transformed AUC_{glcRIA} and $AUC_{glcELISA}$ were statistically significant (R^2 = 0.42, 0.25, and 0.20 and P = 0.001, 0.001, and 0.004, respectively; Figure 2C–E). However, the levels of glucagon at preloading were undetectable by ELISA, even if those measured by RIA were detected in 17 of 40 (42.5%) patients. The peak levels and logarithm-transformed AUC levels of glucagon measured by RIA and ELISA were also decreased in some patients.

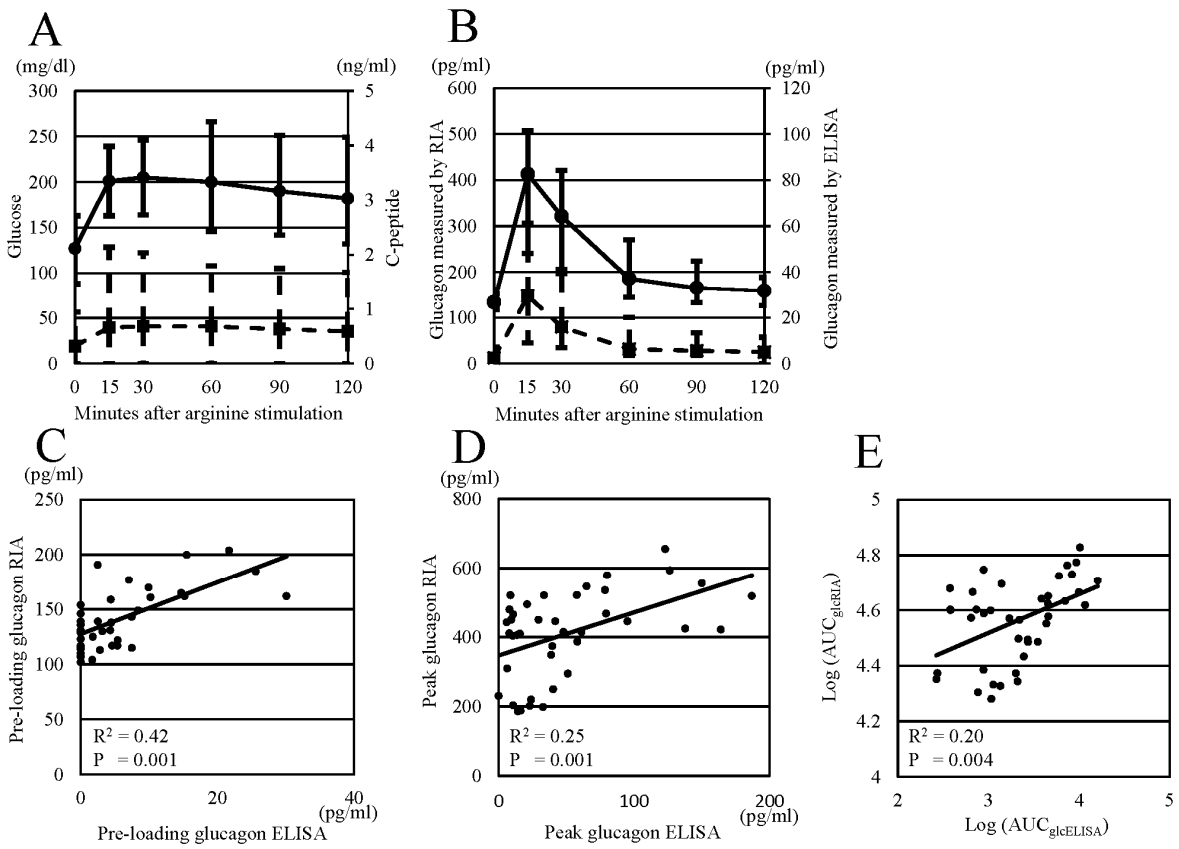

Figure 2. Trends in responses to arginine stimulation and coefficients of plasma glucagon measurements by RIA or ELISA. A: Trends in plasma glucose (solid line with circles) and serum C-peptide (dashed line with squares) responses to arginine stimulation. B: Trends in plasma glucagon responses to arginine stimulation measured by RIA (solid line with circles) and ELISA (dashed line with squares). C: Scatterplot of plasma glucagon levels at preloading measured by RIA and ELISA. D: Scatter plot of peak levels of plasma glucagon measured by RIA and ELISA. E: Scatter plot of log(AUC_{glcRIA}) and log($AUC_{glcELISA}$). Solid lines in C–E show approximate lines between each measurement. Log(AUC_{glcRIA}): logarithm-transformed AUC_{glcRIA}; Log($AUC_{glcELISA}$): logarithm-transformed $AUC_{glcELISA}$; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; AUC: area under the curve.

Associations between AUC_{glcRIA} or $AUC_{glcELISA}$ and the dawn index

Of 40 patients who underwent arginine stimulation tests, four patients could not have a

cannula placed in the forearm, and six patients had to discontinue the test during evaluation of the dawn index because of problems with blood collection and were excluded from the analysis. The median (interquartile range) dawn index was 1.7 (1.0–2.8) and was not significantly correlated with AUC_{glcRIA} or $AUC_{glcELISA}$ ($R^2 = 0.03$, $P = 0.39$ and $R^2 = 0.04$, $P = 0.32$, respectively; Figure 3A and 3B).

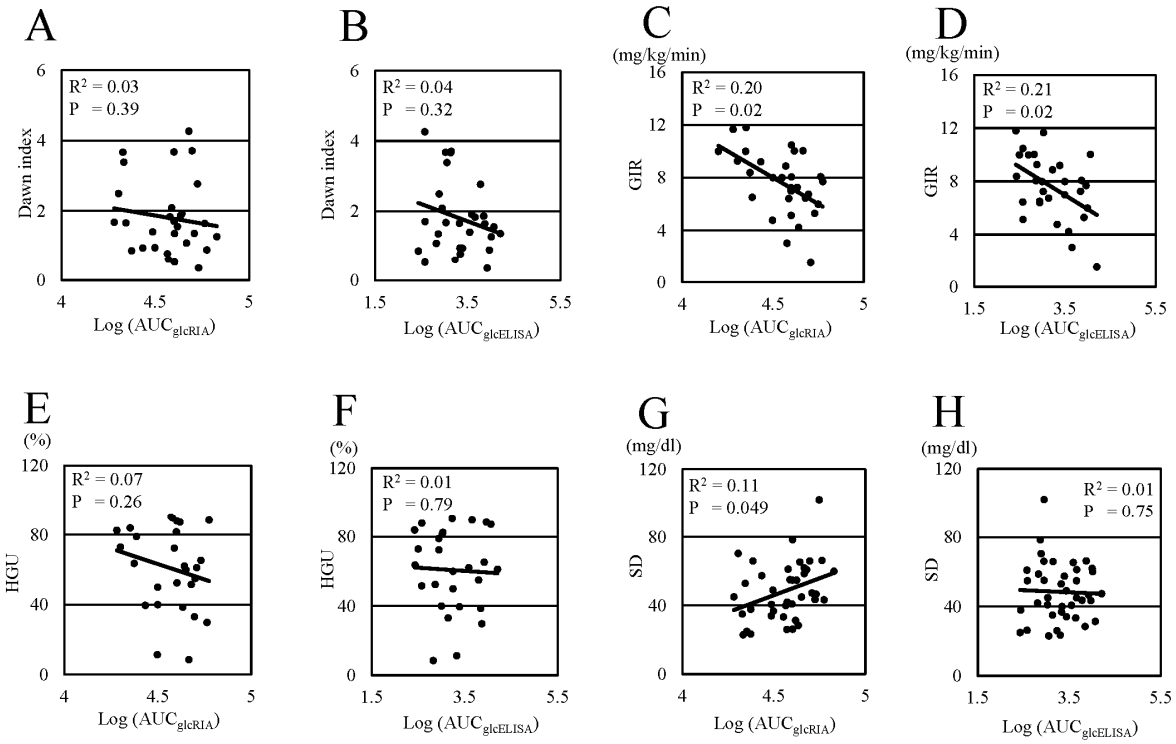

Figure 3. Scatter plots between measurements of glucose metabolism or glycemic variability and log(AUC_{glcRIA}) or log($AUC_{glcELISA}$). A: Scatter plot between the dawn index and log(AUC_{glcRIA}). B: Scatter plot between the dawn index and log($AUC_{glcELISA}$). C: Scatter plot between GIR and log(AUC_{glcRIA}). D: Scatter plot between GIR and log($AUC_{glcELISA}$). E: Scatter plot between HGU and log(AUC_{glcRIA}). F: Scatter plot between HGU and log($AUC_{glcELISA}$). G: Scatter plot between SD and log(AUC_{glcRIA}). H: Scatter plot between SD and log($AUC_{glcELISA}$). Solid lines show approximate lines for

each measurement. The dawn index was defined as the ratio of the average insulin dose delivered to maintain euglycemia (80–110 mg/dL) with an artificial pancreas between 04:00 to 08:00 to that between 00:00 to 04:00. GIR: glucose infusion rate during hyperinsulinemic euglycemic clamp tests; HGU: hepatic glucose uptake evaluated by clamp oral glucose loading tests, as previously described [42]; SD: standard deviation of glucose levels in 96 h, as evaluated by continuous glucose monitoring; Log(AUC_{glcRIA}): logarithm-transformed AUC_{glcRIA}; Log($AUC_{glcELISA}$): logarithm-transformed $AUC_{glcELISA}$; RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbent assay; AUC: area under the curve.

I also analyzed the correlations between the dawn index and fasting levels of glucose-related hormones (i.e., GH, IGF-1, ACTH, cortisol, GLP-1, and somatostatin). There were no significant correlations among these parameters (Figure 4).

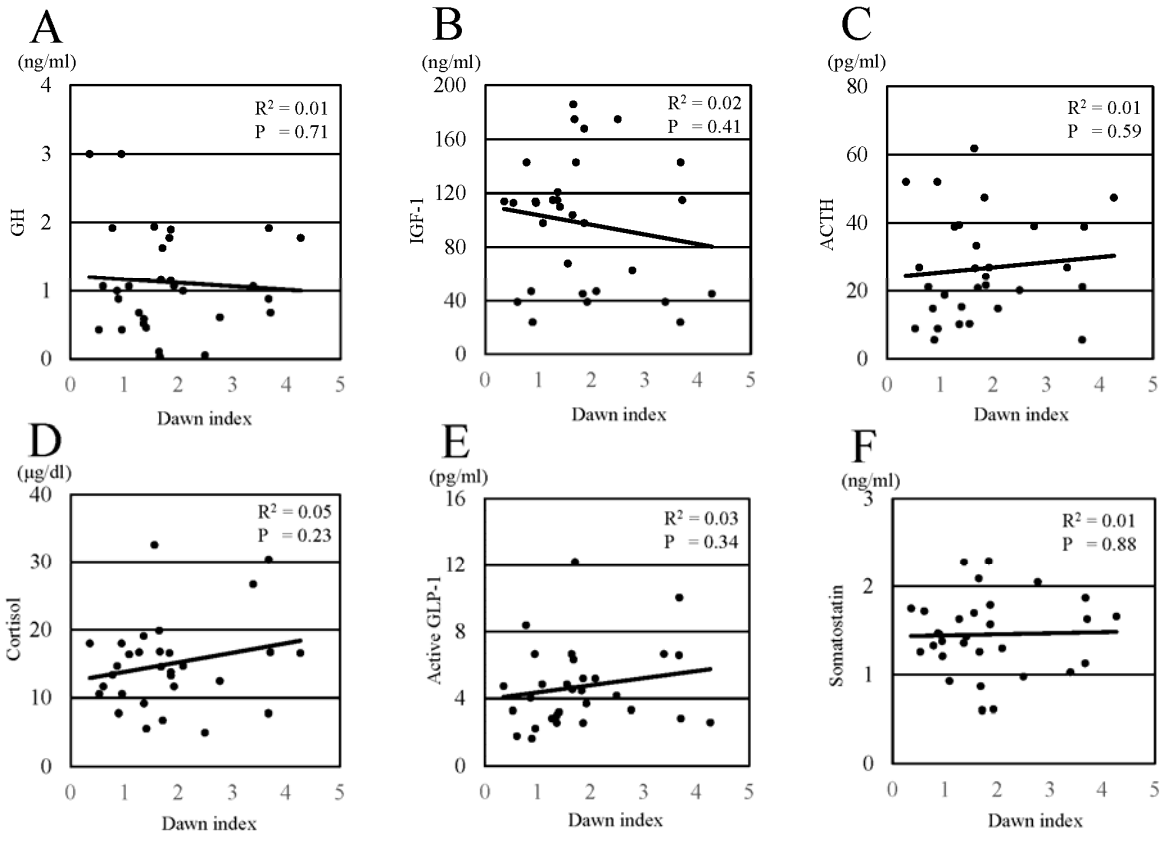

Figure 4. Scatter plots between the dawn index and fasting levels of glucose-related

hormones. A: GH, B: IGF-1, C: ACTH, D: cortisol, E: active GLP-1, F: somatostatin. Solid lines show approximate lines for each measurement. The dawn index was defined as the ratio of the average insulin dose delivered to maintain euglycemia (80–110 mg/dL) with an artificial pancreas between 04:00 and 08:00 to that between 00:00 and 04:00. GH: growth hormone; IGF-1: insulin-like growth factor-1; ACTH: adrenocorticotropic hormone; GLP-1: glucagon-related protein-1.

AUC_{glcRIA} and $AUC_{glcELISA}$ were associated with GIR, but not HGU, as evaluated by hyperinsulinemic euglycemic clamp and clamp oral glucose loading tests

During clamp oral glucose loading tests, GIR in two patients reached zero after glucose loading, and their HGU values were then excluded from analysis. The median (interquartile range) GIR during hyperinsulinemic euglycemic clamp assays and HGU evaluated by clamp oral glucose loading tests were 7.3 (6.4–9.2) mg/kg/min and 65.3% (40.0–87.3%), respectively. The AUC_{glcRIA} and $AUC_{glcELISA}$ were significantly negatively correlated with GIR ($R^2 = 0.20$, $P = 0.02$ and $R^2 = 0.21$, $P = 0.02$, respectively; Figure 3C and 3D) but not with HGU ($R^2 = 0.07$, $P = 0.26$ and $R^2 = 0.01$, $P = 0.79$, respectively; Figure 3E, 3F). Considering confounding variables, multiple regression analysis demonstrated that none of the variables (age, sex, BMI, and AUC_{glcRIA}) were significant predictors of GIR in model 1. However, age and $AUC_{glcELISA}$ were significant predictors of GIR in model 2 (Table 2).

Table 2. Multiple regression analysis of GIR during hyperinsulinemic euglycemic clamp tests

Model 1

Variables	Correlation coefficient	Confidential interval	*P* value
Age (years)	-0.07	-0.16–0.01	0.09
Sex (M/F)	-0.88	-3.31–1.55	0.46
BMI (kg/m^2)	-0.09	-0.59–0.41	0.72
Log (AUC_{glcRIA})	-4.13	-12.85–4.58	0.33

Model 2

Variables	Correlation coefficient	Confidential interval	*P* value
Age (years)	-0.08	-0.15–-0.02	0.014
Sex (M/F)	0.49	-1.78–2.78	0.65
BMI (kg/m^2)	-0.22	-0.63–0.20	0.72
Log ($AUC_{glcELISA}$)	-2.19	-4.21–-0.17	0.035

Sex: male (M) = 0, female (F) = 1; Log(AUC_{glcRIA}): logarithm-transformed AUC_{glcRIA}; Log($AUC_{glcELISA}$): logarithm-transformed $AUC_{glcELISA}$; GIR: glucose infusion rate during

hyperinsulinemic euglycemic clamp tests; BMI: body mass index calculated by weight in kilograms divided by height in meters squared; AUC: area under the curve; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay.

I also compared GIR, HGU, and baseline characteristics between patients with glucagon hypo- or hyperreactivity who could be evaluated GIR and HGU. Only GIR was significantly higher in patients with glucagon hyporeactivity than those with glucagon hyperreactivity (Table 3).

Table 3. Clinical characteristics, GIR, and HGU in patients with glucagon hyporeactivity or hyperreactivity

	Glucagon hyporeactivity (n = 11)	**Glucagon hyperreactivity (n = 17)**	***P***
Age (years)	46 (31–66)	62 (44–72)	0.28
Female	4 (36.3%)	9 (52.9%)	0.48
BMI (kg/m^2)	22.3 (19.8–24.0)	21.0 (19.3–22.5)	0.64
Diabetes duration (years)	1.9 (0.5–11.6)	2.6 (0.1–8.2)	0.80
HbA1c (%)	8.6 (7.5–14.8)	8.2 (7.2–9.2)	0.19
(mmol/mol)	70 (58–138)	66 (55–77)	0.19

Fasting serum C-peptide (ng/mL)	0.33 (0–1.08)	0.17 (0–0.94)	0.71
Estimated GFR (mL/min/1.73 m^2)	92.7 (80.0–120.9)	85.9 (69.6–102.9)	0.20
Insulin treatment			
MDI/CSII	9/2	14/3	0.67
Total daily insulin dose per weight (units/day/kg)	0.65 (0.35–0.78)	0.49 (0.31–0.73)	0.40
Basal/bolus ratio	0.53 (0.36–1.27)	0.40 (0.28–0.60)	0.12
GIR (mg/min/kg)	9.24 (7.02–11.67)	6.75 (5.14–8.08)	0.03
HGU (%)	82.5 (40.0–84.0)	62.9 (42.4–84.8)	0.22

Data are presented as n, n (%), or median (interquartile range). A peak level of glucagon evaluated by RIA during arginine stimulation tests of equal to or more than 300 pg/mL was defined as glucagon hyperreactivity, and the one lower than 300 pg/mL was defined as glucagon hyporeactivity [38]. BMI: body mass index calculated by weight in kilograms divided by height in meters squared; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate calculated using the following formula [36]: estimated GFR (mL/min/1.73 m^2) = 194 × (serum creatinine level, mg/dL)$^{-1.094}$ × (age, years)$^{-0.287}$ (× 0.739 if the patient was female); MDI: multiple daily injection; CSII: continuous subcutaneous insulin infusion; GIR: glucose infusion rate during hyperinsulinemic euglycemic clamp; HGU: hepatic glucose uptake evaluated by clamp oral glucose loading tests, as previously described [42].

I also analyzed correlations between HGU and fasting levels of glucose-related

hormones. HGU was significantly correlated with fasting cortisol levels ($R^2 = 0.28$, $P = 0.003$) and was not correlated with any other glucose-related hormones (Figure 5).

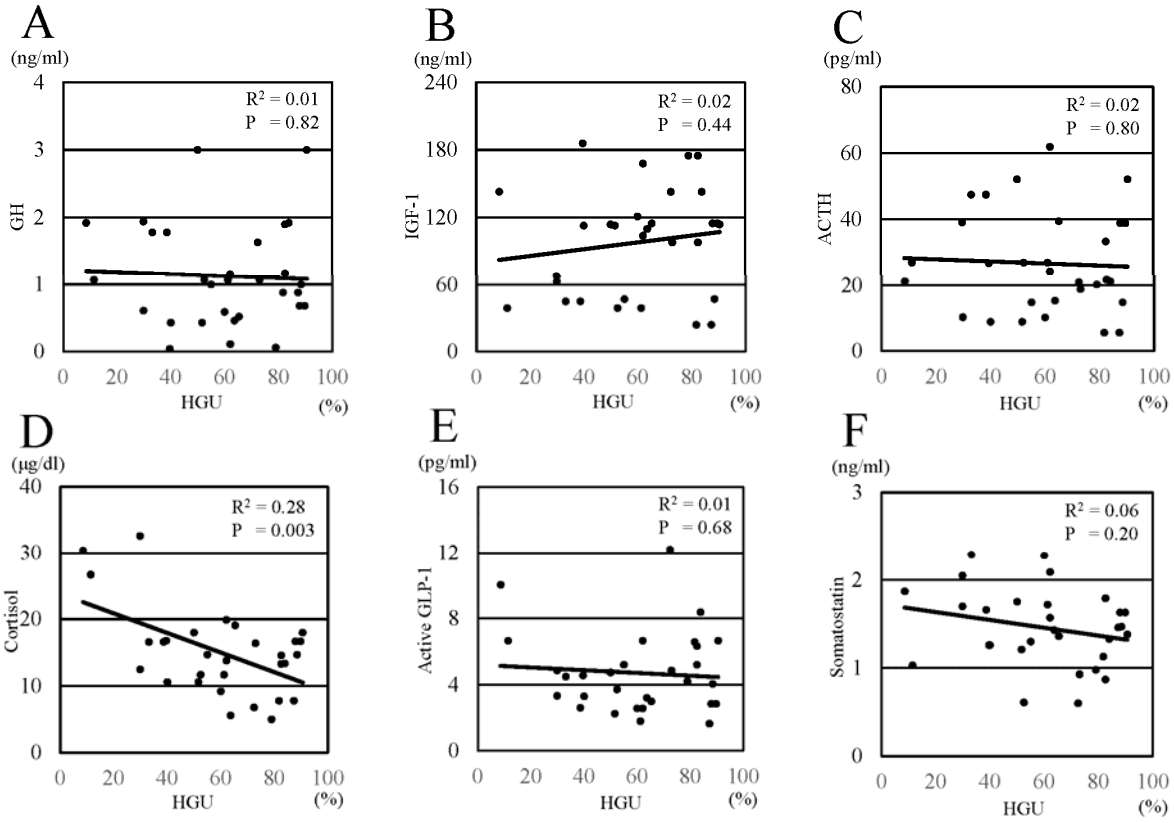

Figure 5. Scatter plots between HGU evaluated by clamp oral glucose loading tests and fasting levels of glucose-related hormones. A: GH, B: IGF-1, C: ACTH, D: cortisol, E: active GLP-1, F: somatostatin. Solid lines show approximate lines for each measurement. The HGU was evaluated by clamp oral glucose loading tests, as previously described [42]. HGU: hepatic glucose uptake; GH: growth hormone; IGF-1: insulin-like growth factor-1; ACTH: adrenocorticotropic hormone; GLP-1: glucagon-related protein-1.

AUC_{glcRIA}, but not $AUC_{glcELISA}$, was associated with glycemic variability evaluated by CGM

The median (interquartile) values for the average, SD, MAGE, M-value, hyperglycemic time, and hypoglycemic time of glucose levels, as evaluated by CGM, within 96 h were 148.4 (126.1–175.9) mg/dL, 46.7 (35.1–60.1) mg/dL, 111.4 (90–132.2), 18.8 (11.8–48.0) mg/dL, 465.0 (216.7–893.3) min/day, and 15.0 (0–120.0) min/day, respectively. Of these measurements, SD was significantly correlated with logarithm-transformed AUC_{glcRIA} positively ($R^2 = 0.11$, $P = 0.049$) but not with logarithm-transformed $AUC_{glcELISA}$ ($R^2 = 0.01$, $P = 0.75$; Figure 3G and 3H). Other measurements of glycemic variability were not significantly correlated with AUC_{glcRIA} or $AUC_{glcELISA}$ (Figure 6).

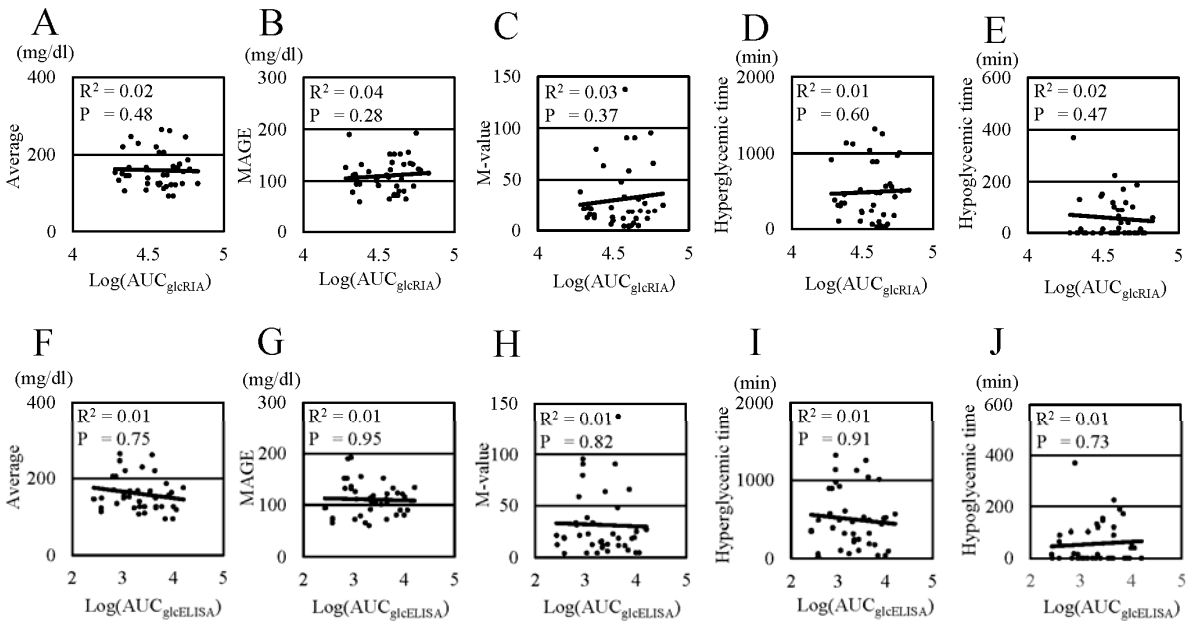

Figure 6. Scatter plots between measurements of glucose variability, except SD (average, MAGE, M-value, hyperglycemic time, and hypoglycemic time), as evaluated by CGM in 96 h and log(AUC_{glcRIA}) (A–E) or log($AUC_{glcELISA}$) (F–J). Solid lines show approximate lines for each measurement. MAGE and M-value were calculated as previously described

[43, 44]. Hyperglycemic and hypoglycemic times were defined as the average number of minutes during which a patient's glucose levels were higher than 180 or less than 70 mg/dL in a day, respectively. CGM: continuous glucose monitoring; Log(AUC_{glcRIA}): logarithm-transformed AUC_{glcRIA}; Log($AUC_{glcELISA}$): logarithm-transformed $AUC_{glcELISA}$; RIA: radioimmunoassay, ELISA: enzyme-linked immunosorbent assay; AUC: area under the curve; MAGE: mean amplitude of glycemic excursion.

Discussion

The results of this study can be summarized as follows: 1) the levels of glucagon measured by RIA and ELISA kits showed almost the same trends, although these glucagon levels decreased in some patients; 2) in terms of insulin sensitivity, the GIR evaluated by hyperinsulinemic euglycemic clamp was significantly correlated with AUC_{glcRIA} and $AUC_{glcELISA}$ negatively, age and $AUC_{glcELISA}$ were independent factors for the GIR in multivariate analysis, and the GIR was significantly higher in patients with glucagon hyporeactivity than that with hyperreactivity; 3) in terms of dawn phenomenon and HGU, the dawn index and HGU did not significantly correlate with AUC_{glcRIA} and $AUC_{glcELISA}$, whereas HGU significantly correlated with cortisol levels; 4) in terms of glucose variability, SD of glucose levels evaluated by CGM significantly correlated AUC_{glcRIA} positively but not with $AUC_{glcELISA}$.

The glucagon response to arginine stimulation involves the reproducible and complementary pancreatic endocrinological functions of both alpha and beta cells [45,

46]. In the present study, trends in the glucagon response to arginine stimulation measured by RIA or ELISA were generally similar, and glucagon levels at preloading, peak, and logarithm-transformed AUC measured by RIA or ELISA were significantly correlated. However, these measurements varied in some patients, as shown in Figure 2. As mentioned above, measurement of glucagon with the RIA kit uses polyclonal antibodies against the glucagon C-terminal region, and these antibodies crossreact with other proglucagon fragments that also contain the C-terminal region, whereas double-sandwich ELISA kits use monoclonal antibodies against both the C- and N-terminal regions of glucagon and measure glucagon concentrations with much lower crossreactivity against proglucagon fragments other than glucagon (1–29) [34]. In a previous report, secretion of GLP-1, which is one of the proglucagon fragment, was also stimulated by arginine loading [33]. The discrepancy between AUC_{glcRIA} and $AUC_{glcELISA}$ in some patients suggested that the differences between the responses of glucagon (1–29) to arginine stimulation and those of other proglucagon fragments. I did not measure the levels of other proglucagon fragments in this study. Further studies are needed to evaluate the responses of other proglucagon fragments.

Regarding associations between glucagon and insulin sensitivity, previous studies have shown that increased fasting levels of glucagon or glucagon responses to

arginine stimulation can contribute to worsening insulin sensitivity in healthy individuals or patients with impaired glucose tolerance [47, 48]. The primary mechanism which alpha cells adapt to insulin sensitivity is mainly thought to be “paracrinopathy,” which designates the loss of tonic restraint normally exerted by a high local concentration of insulin on pancreatic alpha cells [49]. In our study, GIR during hyperinsulinemic euglycemic clamp tests was significantly negatively correlated with AUC_{glcRIA} and $AUC_{glcELISA}$. Moreover, age and $AUC_{glcELISA}$ were independent variables for GIR in multiple regression analysis. These results suggested that pancreatic alpha cell function independently contributed to insulin sensitivity, also in patients with type 1 diabetes, whose beta cell function is compromised. Potential adaptive mediators such as nutrients (branched amino acid and free fatty acids) [50], incretin hormones, and adipocytokines can be considered. Indeed, clinical data confirm that GLP-1 ameliorates insulin sensitivity [51, 52]. The stress effects of obesity may also involve alpha cell function [53]. However, I showed that $AUC_{glcELISA}$ was an independent variable of GIR in multiple regression analysis, suggesting that alpha cell function independently contributes to insulin sensitivity.

In terms of dawn phenomenon, I did not find any correlations between the dawn index and AUC_{glcRIA} or $AUC_{glcELISA}$, which means pancreatic alpha cell function appeared

not to be related to the dawn phenomenon. In previous studies, circadian variations in counter-regulatory hormones (e.g., GH, IGF-1, and cortisol) could affect endogenous glucose production and cause the observed increase in blood glucose levels [18, 54]. In a study with healthy individuals, endogenous glucose production was found to increase as glucagon concentrations increased in the morning [55]. However, subsequent studies concluded that there were no associations between the glucagon concentration and the dawn phenomenon [54, 56]. My study's results were inconsistent with these previous studies.

Cortisol has been shown to play a pivotal role in the stimulation of HGU [57]. The significant correlation between HGU evaluated by clamp oral glucose loading tests and fasting levels of cortisol in my study appeared to indicate the pathophysiological effects of cortisol on hepatic glucose metabolism. Interestingly, hepatic glucose production is rapidly stimulated by the physiological rise in glucagon, which is entirely attributable to enhancement of glycogenolysis [58]. Other previous studies in animals have reported that increasing intraportal infusion of glucagon decreases HGU [59]. Although a study in patients with type 2 diabetes also suggested the association between glucagon and HGU [60], another report in patients with insulin-dependent diabetes could not find any association between glucagon response to oral glucose loading and HGU

[61]. In the present study, I also did not find any significant correlations between HGU and AUC_{glcRIA} or $AUC_{glcELISA}$. These results indicated that pancreatic alpha cell function was not associated with HGU in patients with type 1 diabetes, which is inconsistent with the prior study.

Emerging evidence suggests that glycemic variability contributes to adverse clinical outcomes [62]. Notably, glycemic instability is caused by a deficiency of intrinsic insulin secretion and the paradoxical behaviors of alpha cells during glycemic changes [49], i.e., a deficient glucagon response to hypoglycemia [63] and an inappropriately high glucagon response to hyperglycemia [64]. A previous report showed a positive correlation between glucagon responses to arginine stimulation and several parameters of glycemic variability evaluated by CGM in patients with type 1 diabetes [65]. However, the plasma glucagon levels in these previous reports were measured with RIA kits. In my study, AUC_{glcRIA} was significantly correlated with the SD of glucose levels, similar to the findings of a previous report. In contrast, $AUC_{glcELISA}$ was not correlated with the measurement of glycemic variability. As mentioned above, a recent study suggested that the trend of glucagon levels measured by ELISA kit differed from that measured by RIA kit during meal tolerance test or oral glucose tolerance test [34]. Indeed, proglucagon fragments such as the glicentin and oxyntomodulin, are secreted from the intestine in

response to feeding [66, 67]. The discrepancy between the correlation of glycemic variability with AUC_{glcRIA} and $AUC_{glcELISA}$ in the present study appeared to indicate that proglucagon fragments other than glucagon (1-29) could contribute to glycemic variability.

My study had several limitations. First, this was a cross-sectional study performed at a single national center, and the sample size was small. Prospective studies performed at multiple centers with large sample sizes are needed in order to confirm my results. Second, I evaluated HGU with clamp oral glucose loading tests during hyperinsulinemic euglycemic clamp tests, as described previously [42]. I chose this method because I could evaluate GIR and HGU continuously during hyperinsulinemic euglycemic clamp test and because the use of radioactive tracers for human studies is limited in Japan. Although the reliability of this method was confirmed in a previous report [68], I should perform the direct method to more accurately evaluate HGU.

In conclusion, I found that pancreatic alpha cell function contributed to insulin sensitivity but did not affect HGU and glycemic variability including the dawn phenomenon, in patients with type 1 diabetes. The relationships between pancreatic alpha cell function and glycemic variability could be affected by the purity of glucagon assays. These data provide an important context for the multifactorial role of glucagon in glucose

metabolism in patients with type 1 diabetes.

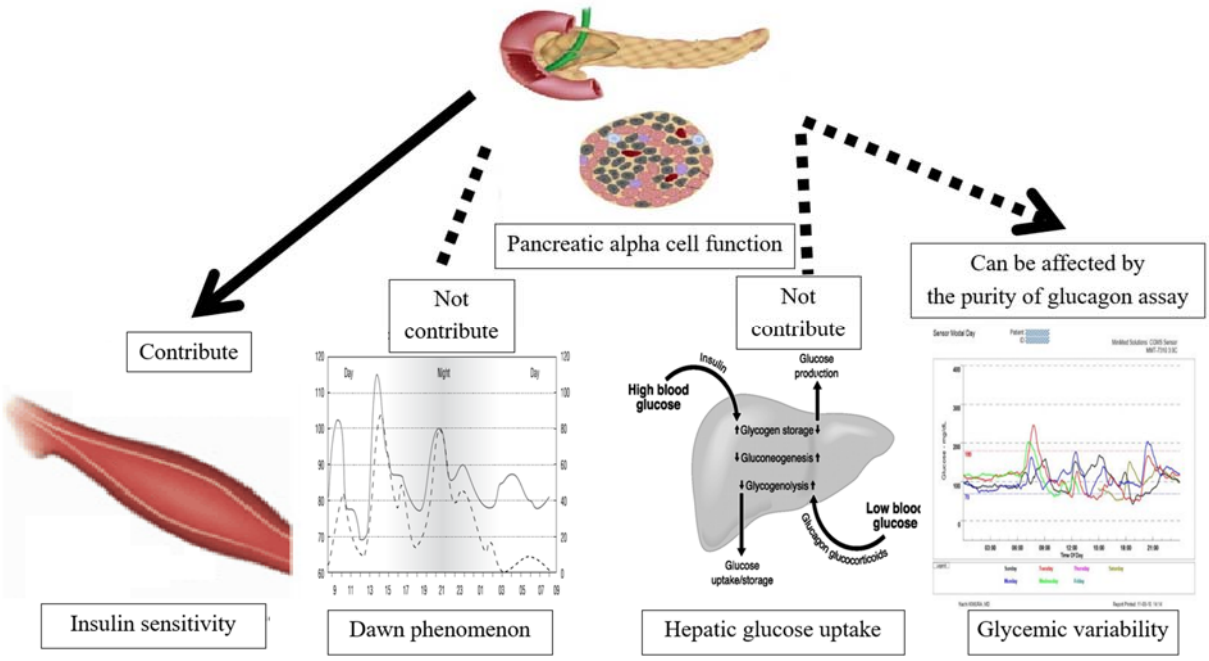

Figure S5. Schema of this study's conclusion. In patients with type 1 diabetes, pancreatic alpha cell function contributes to insulin sensitivity but do not to dawn phenomenon, hepatic glucose uptake, and glycemic variability. The relationship between pancreatic alpha cell function and glycemic variability can be affected by the purity of glucagon assay.

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