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ORIGINAL ARTICLE

Contribution of pancreatic α-cell function to insulin sensitivity and glycemic variability in patients with type 1 diabetes

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ABSTRACT

Aims/Introduction: To evaluate the contribution of pancreatic α-cell function to the dawn phenomenon, insulin sensitivity, hepatic glucose uptake and glycemic variability in patients with type 1 diabetes.

Materials and Methods: In 40 patients with type 1 diabetes, arginine stimulation tests were carried out, and the area under the curve (AUC) of glucagon was measured using radioimmunoassays (AUC_{glcRIA}) and enzyme-linked immunosorbent assays ($AUC_{glcELISA}$). The ratio of the insulin dose delivered by an artificial pancreas to maintain euglycemia between 04.00 and 08.00 hours or between 00.00 and 04.00 hours was measured as the dawn index. The glucose infusion rate and hepatic glucose uptake were measured using hyperinsulinemic euglycemic clamp and clamp oral glucose loading tests. Glycemic variability in 96 h was measured by continuous glucose monitoring.

Results: The median dawn index (1.7, interquartile range 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). The median glucose infusion rate (7.3 mg/kg/min, interquartile range 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). The median hepatic glucose uptake (65.3%, interquartile range 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). The standard deviation of glucose levels measured by continuous glucose monitoring 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 α-cell function contributed to insulin sensitivity in patients with type 1 diabetes.

INTRODUCTION

Type 1 diabetes mellitus is characterized by insulin deficiency caused by pancreatic β-cell destruction[1]. Glucagon inappropriately secretes from pancreatic α-cells and can exacerbate hyperglycemia due to paradoxical hyperglucagonemia or lead to severe hypoglycemia as a result of failed counter-regulation in patients with type 1 diabetes[2]. Notably, patients with type 1 diabetes typically show glucose metabolism mechanisms that are different from those of healthy individuals, including reduced insulin sensitivity, acutely increased blood glucose levels between 05.00 and 09.00 hours (called the "dawn phenomenon"), and impaired hepatic glucose uptake (HGU). These characteristics adversely affect glucose homeostasis and can cause micro- or macrovascular complications.

Despite extensive studies, the role of glucagon as a pathophysiological factor remains unclear. In previous studies, glucagon levels were typically measured using conventional radioimmunoassay (RIA) kits. Quantitative assays known as sandwich enzyme-linked immunosorbent assays (ELISAs) have recently been developed. Thus, it is necessary to compare the results of different assay kits in order to confirm the specific contribution of glucagon to glucose metabolism.

Accordingly, in the present study, we aimed to determine the contribution of pancreatic α- cell function evaluated with RIA or ELISA kits to insulin sensitivity, HGU and glycemic

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variability, including the dawn phenomenon, in patients with type 1 diabetes.

METHODS

Study design and patients

The present observational study was carried out at the National Center for Global Health and Medicine in Tokyo, Japan. We examined patients diagnosed with type 1 diabetes who were admitted to our hospital, and met the inclusion criteria and did not meet the exclusion criteria. Inclusion criteria were as follows: patients who were previously diagnosed with type 1 diabetes according to World Health Organization criteria[3] and were aged ≥20 years. Exclusion criteria were as follows: current treatment with steroid hormones or immunosuppressants, pregnant or breastfeeding, estimated glomerular filtration rate (GFR) of <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[4]: 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). 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, 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.

Arginine stimulation test

On admission, each patient underwent arginine stimulation tests to evaluate their pancreatic α-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 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 administered 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 <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 <20 and <15%, respectively) and sandwich ELISA (Mercodia AB, Uppsala, Sweden; the intra- and interassay coefficients of variation were 7.3–9.4% and 7.5–8.5%, respectively). 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 ≥300 pg/mL was evaluated as glucagon hyperreactivity, whereas that of <300 pg/mL was evaluated as glucagon hyporeactivity, as previously reported[5].

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

After the arginine stimulation test, we 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 hours. At 20.00 hours, 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, 250 U in 500 mL saline; Eli Lily and Company, Indianapolis, IN, USA) in accordance with an algorism to maintain blood glucose levels within the range of 80–110 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[6]. We evaluated changes in insulin requirements from 00.00 to 08.00 hours as the "dawn phenomenon" using this artificial pancreas. The ratio of the delivered insulin dose average between 04.00 and 08.00 hours to that between 00.00 and 04.00 hours was calculated as the dawn index (Figure S1). To support the relationship between the dawn phenomenon and glucose-related hormones, we also measured levels of the following hormones after patients were kept at rest for 30 min after overnight fasting: growth hormone (GH; Elecsys immunoassay; Roche Diagnostics), insulin-like growth factor-1 (IGF-1; immunoradiometric assay; Fujirebio, Tokyo, Japan), adrenocorticotropic hormone (Elecsys immunoassay; Roche Diagnostics), cortisol (chemiluminescent immunoassay; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA), active glucagon-related protein-1 (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*[7]. At 08.00 hours 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[8]. 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 carried out to evaluate HGU as previously described[9]. 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. The glucose infusion rate 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. After 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). We 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 analysis. To support the relationship between HGU and glucose-related hormones, we also analyzed the correlation between HGU and fasting levels of GH, IGF-1, adrenocorticotropic hormone, cortisol, active GLP-1 and somatostatin.

Assessment of glycemic variability

A total of 24 h 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[10], mean amplitude of glycemic excursions[11], 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 >180 or <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 carried out to analyze the correlations among measurements. Multiple regression analysis was carried out 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}; and model 2 included age, sex, BMI and $AUC_{glcELISA}$. Results with *P*-values of <0.05 were considered statistically significant. All analyses were carried out 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 did not meet the exclusion criteria participated in the present 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 <1.0 ng/mL, suggesting that their β-cell function was severely impaired.

Glucagon response to arginine stimulation measured by RIA or ELISA

Figure 1a,b 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 1a).

Table 1 | Clinical characteristics of the patients included in the 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)
eGFR (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; CSII, continuous subcutaneous insulin infusion; eGFR, estimated glomerular filtration rate calculated using the following formula[4]: 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); HbA1c, glycated hemoglobin; MDI, multiple daily injection.

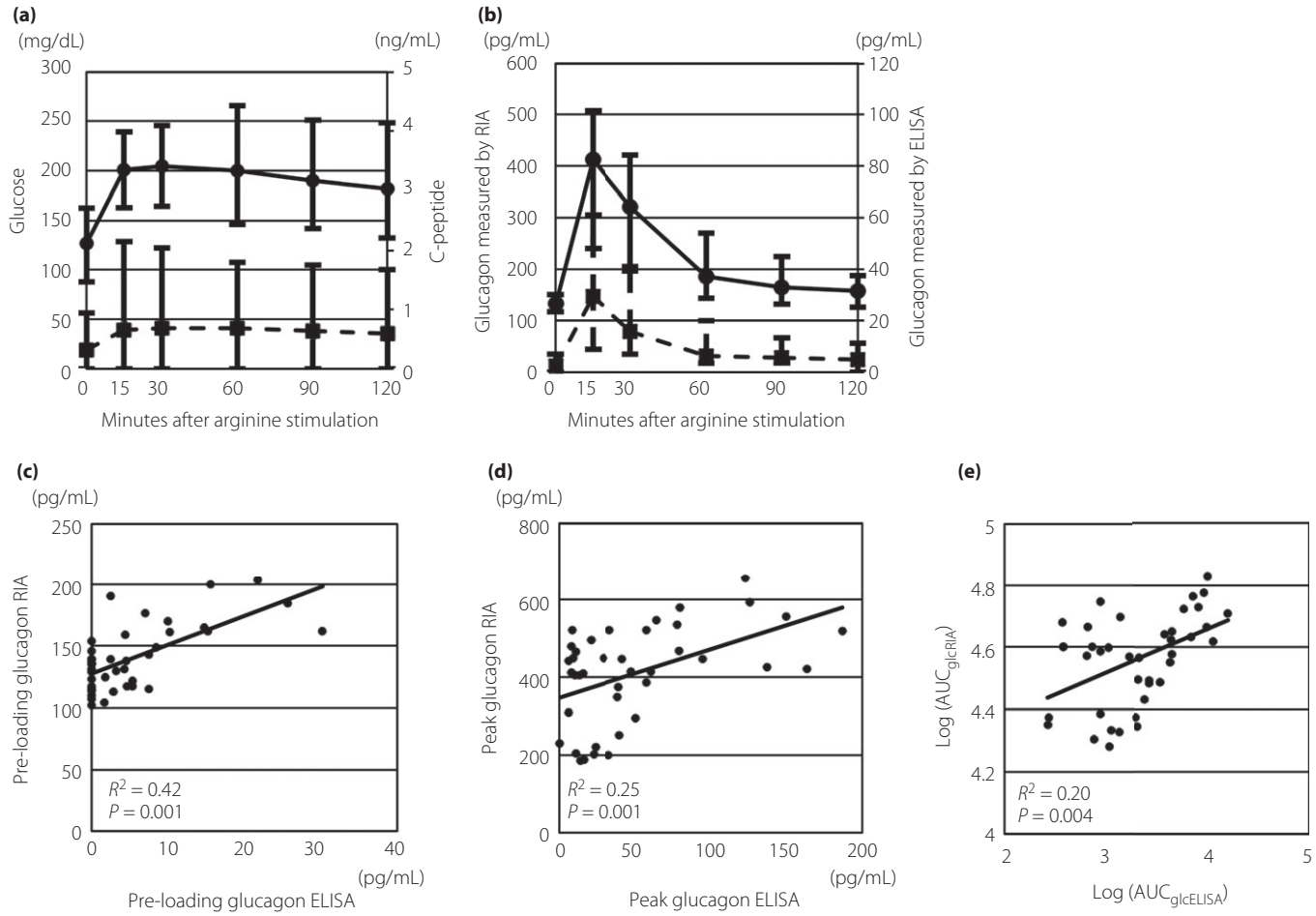

Figure 1 | Trends in responses to arginine stimulation and coefficients of plasma glucagon measurements by radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA). (a) Trends in plasma glucose (solid line with circles) and serum C-peptide (dashed line with squares) responses to arginine 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 logarithm-transformed area under the curve (AUC) of glucagon measured using radioimmunoassays (log[AUC_{glcRIA}]) and logarithm-transformed AUC of glucagon measured using enzyme-linked immunosorbent assays (log [$AUC_{glcELISA}$]). Solid lines in (c–e) show approximate lines between each measurement.

The median (interquartile range) plasma glucagon levels at preloading and peak, as measured by RIA and the AUC_{glcRIA}, were 133.5 pg/mL (117.0–151.5 pg/mL), 413.0 pg/mL (272.5–507.0 pg/mL) and 3.7×10^4 pg/mL·min ($2.6–4.6 \times 10^4$ pg/mL·min), respectively, and those measured by ELISA were 2.5 pg/mL (0–7.0 pg/mL), 32.8 pg/mL (10.7–61.2 pg/mL) and 2.0×10^3 pg/mL·min ($0.8–4.5 \times 10^3$ pg/mL·min), respectively. Trends in the glucagon response to arginine stimulation, as measured by RIA or ELISA, were similar (Figure 1b). 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 1c–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.

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 2a,b). We also analyzed the correlations between the dawn index and fasting levels of glucose-related hormones (i.e., GH, IGF-1, adrenocorticotropic

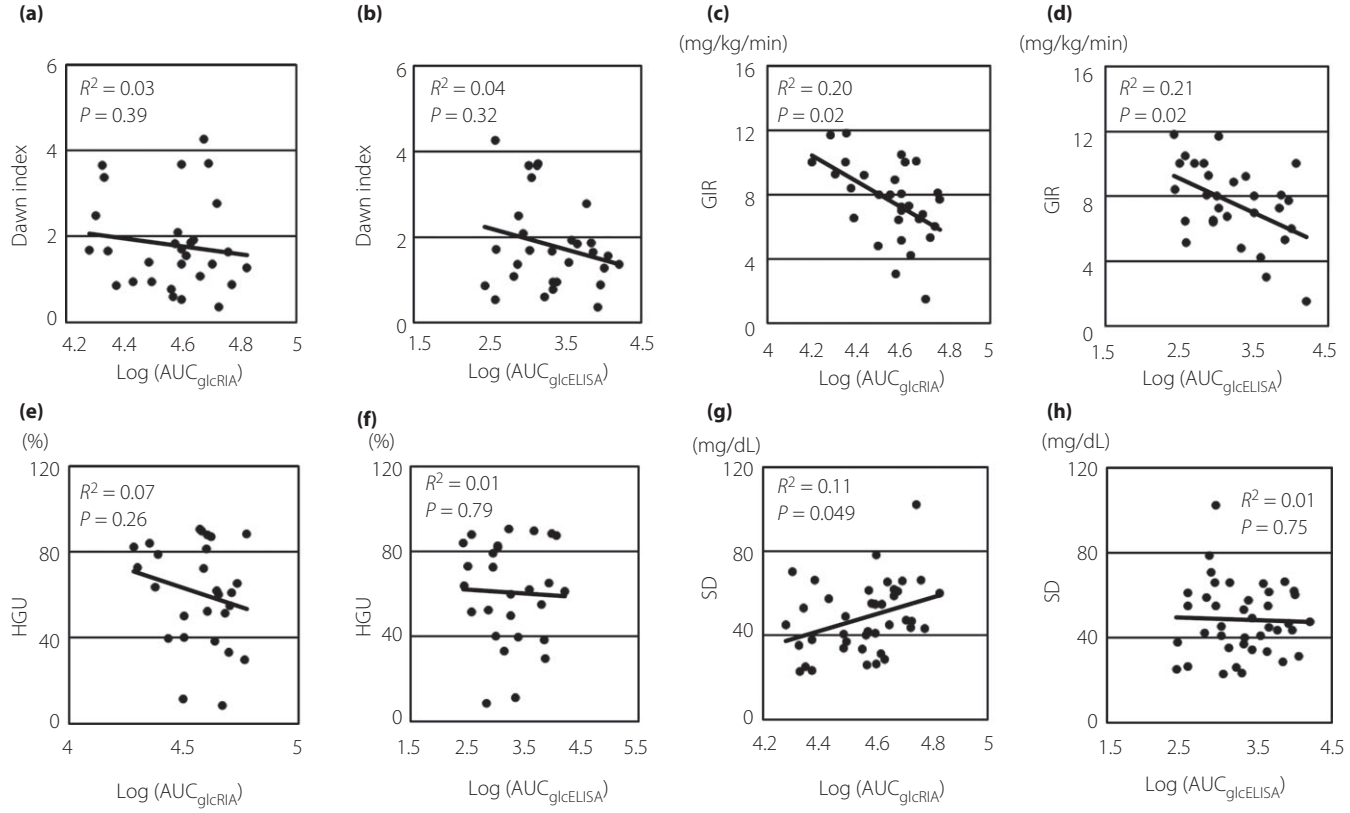

Figure 2 | Scatter plots between measurements of glucose metabolism or glycemic variability and log(AUC_{glcRIA}) or log($AUC_{glcELISA}$). (a) Scatter plot between the dawn index and log(AUC_{glcRIA}). (b) Scatter plot between the dawn index and log($AUC_{glcELISA}$). (c) Scatter plot between GIR and 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 and 08:00 to that between 00:00 and 04:00. AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; GIR, glucose infusion rate during hyperinsulinemic euglycemic clamp tests; HGU, hepatic glucose uptake evaluated by clamp oral glucose loading tests, as previously described[9]; Log($AUC_{glcELISA}$), logarithm-transformed $AUC_{glcELISA}$; Log(AUC_{glcRIA}), logarithm-transformed AUC_{glcRIA}; RIA, radioimmunoassay; SD, standard deviation of glucose levels in 96 h, as evaluated by continuous glucose monitoring.

hormone, cortisol, GLP-1 and somatostatin). There were no significant correlations among these parameters (Figure S2).

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 mg/kg/min (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 2c,d), but not with HGU ($R^2 = 0.07$, $P = 0.26$ and $R^2 = 0.01$, $P = 0.79$, respectively; Figure 2e,f). Considering confounding variables, multiple regression analysis showed 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 S1).

We also compared GIR, HGU and baseline characteristics between patients with glucagon hypo- or hyperreactivity who could be evaluated for GIR and HGU. Only GIR was significantly higher in patients with glucagon hyporeactivity than those with glucagon hyperreactivity (Table 2).

We also analyzed correlations between HGU and fasting levels of glucose-related hormones. Hepatic glucose uptake 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 S3).

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

The median (interquartile) values for the average, SD, mean amplitude of glycemic excursions, M-value, hyperglycemic

Table 2 | Clinical characteristics, glucose infusion rate and hepatic glucose uptake 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
eGFR (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 radioimmunoassay during arginine stimulation tests of ≥300 pg/mL was defined as glucagon hyperreactivity, and that of <300 pg/mL was defined as glucagon hyporeactivity[5]. BMI, body mass index calculated by weight in kilograms divided by height in meters squared; CSII, continuous subcutaneous insulin infusion; eGFR, estimated glomerular filtration rate calculated using the following formula[4]: 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); GIR, glucose infusion rate during hyperinsulinemic euglycemic clamp; HbA1c, glycated hemoglobin; HGU, hepatic glucose uptake evaluated by clamp oral glucose loading tests, as previously described[9]; MDI, multiple daily injection.

time and hypoglycemic time of glucose levels, as evaluated by CGM, within 96 h were 148.4 mg/dL (126.1–175.9 mg/dL), 46.7 mg/dL (35.1–60.1 mg/dL), 111.4 (90–132.2), 18.8 mg/dL (11.8–48.0 mg/dL), 465.0 min/day (216.7–893.3 min/day) and 15.0 min/day (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 2g,h). Other measurements of glycemic variability were not significantly correlated with AUC_{glcRIA} or $AUC_{glcELISA}$ (Figure S4).

DISCUSSION

To the best of our knowledge, this is the first study to report the associations between glucagon response to arginine stimulation measured by RIA or ELISA, and dawn phenomenon, insulin sensitivity, HGU and measurement of glycemic variability in patients with type 1 diabetes. The glucagon response to arginine stimulation involves the reproducible and complementary pancreatic endocrinological functions of both α- and β-cells[12,13]. 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 1. Glucagon (1–29) is produced through processing of proglucagon by proglucagon convertase[14]. In this process, other proglucagon fragments (e.g., oxyntomodulin, glicentin and GLP-1) were also produced. 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. In contrast, double-sandwich ELISA kits use monoclonal antibodies against both the C- and N-terminal regions of glucagon and measure glucagon concentrations with much lower cross-reactivity against proglucagon fragments other than glucagon (1–29)[15]. In a previous report, secretion of GLP-1 was also stimulated by arginine loading[16]. The discrepancy between AUC_{glcRIA} and $AUC_{glcELISA}$ in some patients suggested that the differences between responses of glucagon (1–29) to arginine stimulation and those of other proglucagon fragments. We did not measure the levels of other proglucagon fragments in the present study. Further studies are required to evaluate the responses of other proglucagon fragments.

In terms of 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[17,18]. The mechanisms through which α-cells adapt to insulin sensitivity remain unclear. The primary mechanism is thought to be "paracrinopathy," which designates the loss of tonic restraint normally exerted by a high local concentration of insulin on α-cells[2]. In the present study, GIR during hyperinsulinemic euglycemic clamp tests was significantly negatively correlated with AUC_{glcRIA} and $AUC_{glcELISA}$. Furthermore, age and $AUC_{glcELISA}$ were independent variables for GIR in multiple regression analysis. These results suggested that pancreatic α-cell function

independently contributed to insulin sensitivity, even in patients with type 1 diabetes, whose β-cell function is diminished. Potential adaptive mediators, such as nutrients (branched amino acid and free fatty acids)[19], incretin hormones and adipocytokines, can be considered. Indeed, clinical data show that GLP-1 improves insulin sensitivity[20,21]. The stress effects of obesity might also involve α-cell function[22]. However, we showed that $AUC_{glcELISA}$ was an independent variable of GIR in multiple regression analysis, suggesting that α-cell function independently contributes to insulin sensitivity.

The dawn phenomenon, first reported by Schmidt *et al.*[23], refers to the concept that the levels of blood glucose rise acutely between 04.00 and 08.00 hours, and is typically observed in patients with type 1 diabetes. A previous report showed that the dawn phenomenon could affect overall glycemic control[24], and that we should clarify the etiology of this phenomenon. 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[25,26]. In a study of healthy individuals, endogenous glucose production was found to increase as glucagon concentrations increased in the morning[27]. However, subsequent studies concluded that there were no associations between the glucagon concentration and the dawn phenomenon[26,28]. We also did not find any correlations between the dawn index and AUC_{glcRIA} or $AUC_{glcELISA}$. Thus, pancreatic α-cell function appeared not to be related to the dawn phenomenon.

Cortisol has been shown to play a pivotal role in stimulation of HGU[29]. The significant correlation between HGU evaluated by clamp oral glucose loading tests and fasting levels of cortisol in the present study appeared to show 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[30]. Other previous studies in animals have reported that increasing intraportal infusion of glucagon decreases HGU[31]. Although a study of patients with type 2 diabetes also suggested the association between glucagon and HGU[32], another report of patients with insulin-dependent diabetes could not find any association between glucagon response to oral glucose loading and HGU[33]. In the present study, we also did not find any significant correlations between HGU and AUC_{glcRIA} or $AUC_{glcELISA}$. These results indicated that pancreatic α-cell function was not associated with HGU in patients with type 1 diabetes.

Emerging evidence suggests that glycemic variability contributes to adverse clinical outcomes[34]. Notably, glycemic instability is caused by deficiency of intrinsic insulin secretion and the paradoxical behaviors of α-cells during glycemic changes[2]; that is, a deficient glucagon response to hypoglycemia[35] and an inappropriately high glucagon response to hyperglycemia[36]. 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[37]. However, the plasma glucagon levels in these previous reports were measured with RIA kits. In the present 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. According to a previous report, the trend of glucagon levels measured by an ELISA kit differed from that measured by a RIA kit during the meal tolerance test; the former returned slightly elevated results, whereas the latter produced significantly lower levels[38]. The accuracy of glucagon levels measured by an ELISA kit was confirmed with novel liquid chromatography-high resolution mass spectroscopy. Indeed, proglucagon fragments, such as the glicentin and oxyntomodulin, are secreted from the intestine in response to feeding[39,40]. The discrepancy between the correlation of glycemic variability with AUC_{glcRIA} and $AUC_{glcELISA}$ in the present study appeared to show that proglucagon fragments other than glucagon (1–29) could contribute to glycemic variability.

The present study had several limitations. First, this was an observational study carried out at a single national center, and the sample size was small. Prospective studies carried out at multiple centers with large sample sizes are required in order to confirm the present results. Second, we evaluated HGU with clamp oral glucose loading tests during hyperinsulinemic euglycemic clamp tests, as described previously[9]. We chose this method because we 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[41], we should carry out the direct method to more accurately evaluate HGU.

In conclusion, we found that pancreatic α-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 α-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.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Schematic presentation of changes in insulin requirements between night and morning, as evaluated using an artificial pancreas.

Figure S2 | Scatter plots between the dawn index and fasting levels of glucose-related hormones.

Figure S3 | Scatter plots between hepatic glucose uptake evaluated by clamp oral glucose loading tests and fasting levels of glucose-related hormones.

Figure S4 | Scatter plots between measurements of glucose variability, except standard deviation (average, mean amplitude of glycemic excursions, M-value, hyperglycemic time and hypoglycemic time), as evaluated by continuous glucose monitoring in 96 h and (a–e) logarithm-transformed area under the curve (AUC) of glucagon measured using radioimmunoassays ($\log[AUC_{glcRIA}]$) or (f–j) logarithm-transformed AUC of glucagon measured using enzyme-linked immunosorbent assays ($\log[AUC_{glcELISA}]$).

Table S1 | Multiple regression analysis of glucose infusion rate during hyperinsulinemic euglycemic clamp tests.