

Lack of Association Between Residual Insulin Production and Glucagon Response to Hypoglycemia in Youth With Short Duration of Type 1 Diabetes

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OBJECTIVE—To examine the loss of glucagon response to hypoglycemia and its relationship with residual β -cell function early in the course of type 1 diabetes (T1D) in youth.

RESEARCH DESIGN AND METHODS—Twenty-one youth with T1D duration <1 year (ages 8–18 years, T1D duration 6–52 weeks) underwent mixed-meal tolerance tests (MMTTs) to assess residual β -cell function and hypoglycemic clamps to assess glucagon responses to hypoglycemia. Glucagon responses to hypoglycemia in T1D subjects were compared with those in 12 nondiabetic young adults (ages 19–25 years).

RESULTS—Peak MMTT-stimulated C-peptide levels (range 0.12–1.43) were ≥ 0.2 nmol/L in all but one T1D subject. As expected, the median of glucagon responses to hypoglycemia in the T1D subjects (18 pg/mL [interquartile range 7–32]) was significantly reduced compared with the responses in nondiabetic control subjects (38 pg/mL [19–66], $P = 0.02$). However, there was no correlation between the incremental increase in plasma glucagon during the hypoglycemic clamp and the incremental increase and peak plasma C-peptide level during the MMTT. Similarly, the seven T1D subjects who failed to achieve an increase in glucagon ≥ 12 pg/mL (i.e., 3 SD above baseline values) had C-peptide response ≥ 0.2 nmol/L (0.54–1.12), and the one T1D subject with peak stimulated < 0.2 nmol/L had a 14 pg/mL increase in plasma glucagon in response to hypoglycemia.

CONCLUSIONS—Impaired plasma glucagon responses to hypoglycemia are evident in youth with T1D during the first year of the disease. Moreover, defective and absent glucagon responses to hypoglycemia were observed in patients who retained clinically important residual endogenous β -cell function.

Diabetes Care 36:1470–1476, 2013

There are a number of defects in counterregulatory hormone responses that make patients with type 1 diabetes (T1D) especially vulnerable to hypoglycemia (1). Unlike healthy nondiabetic subjects, patients with T1D

on exogenous insulin are unable to suppress endogenous insulin secretion in response to falling plasma glucose levels, and excess exogenous insulin may result in inappropriately elevated insulin levels. In addition, plasma epinephrine responses

are impaired in intensively treated patients (2,3) as a result of recurrent episodes of biochemical hypoglycemia (4) and at night during sleep (5,6). Perhaps most importantly, plasma glucagon responses to hypoglycemia are completely lost in almost all patients with long-standing T1D (7,8).

Despite their central role in glucose counterregulation, the natural history and pathophysiology of the loss of the responsiveness of the α -cell to hypoglycemia in T1D patients have not been established in adults or children. In youth with T1D, previous studies that examined counterregulatory hormone responses to hypoglycemia were carried out in patients who already manifested absent plasma glucagon responses (9–12) and in subjects shortly after diagnosis in which glucagon response did not differ from those with long-standing disease (13). Siafarikas et al. (14) recently reported the loss of glucagon response to hypoglycemia occurring at a median of 8 months' disease duration in adolescents with T1D. In that study of 28 subjects, roughly one-half of the subjects were diagnosed in the past year; however, disease duration ranged from 0.01 to 9.9 years. Although the β -cell is targeted by autoimmune destruction, the same is not true for the α -cells, as secretion of glucagon is preserved to other stimuli such as mixed-meal ingestion (15) or arginine infusion and may even be exaggerated in response to a mixed-meal feeding (16,17). Therefore, dysregulation of α -cell function rather than α -cell destruction appears to be the cause of the loss of glucagon responses to hypoglycemia in patients with T1D.

The intraislet insulin hypothesis postulates that a reduction in insulin levels within the islet resulting from a decrease in insulin secretion in response to falling plasma glucose levels is needed to stimulate increases in glucagon secretion and circulating plasma glucagon concentrations (1,18). A number of animal and human studies have been conducted that support this hypothesis (1,17,19–24).

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Received 21 August 2012 and accepted 30 November 2012.

DOI: 10.2337/dc12-1697

This article contains Supplementary Data online at <http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc12-1697/-DC1>.

*A full list of the members of the DirecNet Study Group can be found in the APPENDIX.

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The hypothesis also suggests that if patients maintained residual β -cell function, they would retain their ability to mount a glucagon response to hypoglycemia. In order to examine the natural history of the loss of glucagon response to hypoglycemia and its relationship to residual β -cell function early in the course of T1D, we performed one-step hypoglycemic clamps and mixed-meal tolerance tests (MMTTs) in children and adolescents with T1D with a disease duration that ranged between 6 and 52 weeks. The changes in plasma glucagon in our youth with T1D were also compared with those in healthy young-adult subjects who underwent a similar one-step hypoglycemic clamp study.

RESEARCH DESIGN AND METHODS

Diabetic subjects

The study in pediatric subjects with T1D was conducted by the Diabetes Research in Children Network (DirecNet) at five clinical centers. The protocol was approved by the institutional review boards of the participating sites, a protocol review committee, and a data safety monitoring board. Written informed consent was obtained from the parents/guardians, and the child's assent was obtained when appropriate. The study protocol was listed on a Web site (<http://direcnet.jaeb.org/Studies.aspx>). Major eligibility criteria included 1) clinical diagnosis of T1D between 6 and 52 weeks prior to enrollment in the study, 2) positive islet antibody titers, and 3) age ≥ 8.0 to < 19.0 years. Subjects were recruited with plans to have equal distribution among four predefined diabetes duration bins: 6–13, 14–26, 27–39, and 40–52 weeks.

Procedures

The MMTT and hypoglycemic clamp procedures were scheduled to be performed within 2 weeks of one another. The MMTT and hypoglycemic clamp studies were conducted in the clinical research center in the morning after an overnight fast. A blood sample was collected at each study for measurement of HbA_{1c} at the University of Minnesota using the Tosoh A1c 2.2 Plus Glycohemoglobin Analyzer (Tosoh Medics, Foster City, CA) method (25).

MMTT. Participants underwent an MMTT for assessment of residual β -cell function. They were instructed to consume at least 150 g carbohydrates/day

for 3 days prior to the MMTT and to consume only water after midnight on the night prior to testing. Subjects on injection therapy were instructed to administer their usual basal insulin dose(s) on the night before or on the morning of the study but to hold their morning dose of short- and intermediate-acting insulin. Subjects on continuous subcutaneous insulin infusion remained on their usual basal rates for the duration of the study. Prior to initiation of the study, blood glucose was targeted to be between 70 and 200 mg/dL. An indwelling catheter was placed for collection of venous samples. A dose of Boost High Protein Nutritional Energy Drink (Mead-Johnson) mixed meal at 6 mL/kg (maximum dose 360 mL) was given at 0 min. Blood samples were obtained for measurement of plasma glucose, C-peptide, and glucagon at –10, 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 min. A split duplicate sample for quality control was collected at either 0 or 240 min.

One-step hypoglycemic clamps in T1D subjects.

For the one-step hyperinsulinemic-hypoglycemic clamp study, subjects were instructed to consume only water after midnight on the night prior to the study. As with the MMTT, subjects on injection therapy were instructed to administer their usual basal insulin dose(s) on the night before or on the morning of the study, and subjects on continuous subcutaneous insulin infusion remained on their usual basal rates for the duration of the study. Bolus doses of rapid-acting insulin analogs were held the morning of the test. On arrival to the clinical research center, an intravenous catheter was placed for administration of insulin and glucose. A second intravenous catheter was placed in a distal arm or hand vein; this arm was gently heated, allowing for sampling of arterialized venous blood. A continuous intravenous infusion of regular insulin was started at 2.0 mU/kg/min and continued for the duration of the study. A variable infusion of 10–20% dextrose solution was adjusted to achieve target glucose levels during the euglycemic and hypoglycemic phases of the study. Plasma glucose levels were measured every 5 min throughout the clamp procedure. Plasma glucose was initially clamped at ~ 95 mg/dL (range 90–100) for 60 min; the dextrose infusion was then decreased, and plasma glucose was allowed to reach the hypoglycemic target (~ 55 mg/dL [range 50–60]) during the 60-min hypoglycemic phase of the study. Blood samples

were obtained for measurement of plasma glucagon and catecholamine levels (reported separately) at 0, 30, 45, and 60 min during the euglycemic phase and at 15, 30, 45, and 60 min of the hypoglycemic phase of the study. Split duplicate samples for quality control were collected at either the beginning of the euglycemic phase or the end of the hypoglycemic phase.

One-step hypoglycemic clamps in non-diabetic subjects.

Studies in 12 nondiabetic young adults between 19 and 25 years of age (mean \pm SD 23.3 \pm 2.0 years) were performed between 2009 and 2010 at the Washington University School of Medicine (St. Louis, MO) under a protocol approved by its institutional review board. Subjects were studied in a similar manner using the one-step hypoglycemic clamp technique described above. Blood was drawn for measurement of plasma glucagon levels every 30 min. Extra plasma glucagon samples from this study were stored at -80°C . For comparison with the results in our pediatric subjects, these stored samples were reassayed in the DirecNet Central Laboratory using the assays described below.

Laboratory procedures

Blood samples were stored frozen at -70°C prior to shipping. Plasma glucagon concentrations were measured at the DirecNet Central Laboratory (University of Minnesota). Glucagon was measured by a radioimmunoassay (Linco Research, St. Charles, MO) with the primary antibody from guinea pig and the secondary antibody from goat. The lower limit of detection was 20 pg/mL (6 pmol/L), and the median coefficient of variation was 9% from quality-control samples collected in this study. C-peptide concentrations were measured at Northwest Lipid Metabolism and Diabetes Research Laboratories (Seattle, WA) using Tosoh AIA 1800. The lower limit of detection was 0.05 ng/mL (0.0167 nmol/L), and the median coefficient of variation was 6.5%.

Statistical methods

The sample size of 25 for diabetic subjects was a convenience sample with a goal of having approximately equal numbers of subjects in four diabetes duration bins: 6–13, 14–26, 27–39, and 40–52 weeks. Twenty-five of the 27 enrolled subjects completed both MMTT and hypoglycemic clamp procedures. However, four of the subjects studied had baseline glucagon values < 20 pg/mL, the lower limit of detection for the assay,

and were excluded from further analysis because the glucagon response to hypoglycemia could not be accurately evaluated. (Data from the excluded subjects can be found in Supplementary Table 1.) Results were similar when the four subjects were included in analysis, and a value of 20 pg/mL was imputed for the baseline glucagon values below detection (data not shown).

The primary analysis was the examination of the relationship between residual β -cell function measured by peak stimulated C-peptide level during MMTT and the glucagon response to hypoglycemia measured by rise in plasma concentrations during the hypoglycemic clamp study. Outcome measures included peak stimulated C-peptide concentration during the MMTT, incremental C-peptide area under the curve (AUC), and clinically important C-peptide response, defined as peak stimulated C-peptide concentration >0.2 nmol/L (26). Rise in glucagon was defined as the peak glucagon achieved during the hypoglycemic portion of the clamp minus the glucagon level obtained at the end of the euglycemic phase of the clamp (Supplementary Table 1). The SD of baseline glucagon levels (based on the coefficient of variation of split duplicate plasma glucagon measurements) was 4 pg/mL. A plasma glucagon response to hypoglycemia was defined as a rise in plasma glucagon concentrations that was ≥ 12 pg/mL or >3 times the SD above baseline concentrations. An absent glucagon response was defined as a rise in plasma glucagon levels <4 pg/mL. C-peptide concentration at the start of the MMTT was considered baseline, and plasma glucagon levels after 60 min of euglycemic hyperinsulinemia were used as the baseline values during hypoglycemic clamps. Incremental glucagon AUC was calculated as the change in glucagon during the hypoglycemic portion of the clamp with the 60-min euglycemic sample serving as the baseline value. Spearman correlations were computed for peak C-peptide, duration of diabetes, and change in plasma glucagon level with hypoglycemia to peak C-peptide, incremental C-peptide AUC, and duration of diabetes. Wilcoxon rank sum tests were performed for comparison of the plasma glucagon concentrations and incremental AUC in diabetic subjects with those in control subjects. Two-sample *t* test was used to compare the nadir glucose levels in diabetic subjects with those in control subjects.

RESULTS

Subject characteristics

The 21 evaluable diabetic subjects were between the ages of 9 and 18 years (13.3 ± 2.6). Mean HbA_{1c} at first test was $6.8 \pm 0.8\%$, with 90% of the subjects on injection therapy. Eight (38%) were female, and 13 (62%) were Caucasian. Only one patient reported ever having an episode of severe hypoglycemia, defined as requiring assistance, and this did not occur during the week prior to the clamp study. There was relatively even distribution among the four disease duration bins, with four subjects with duration of diabetes between 6 and 13 weeks, six between 14 and 26 weeks, six between 27 and 39 weeks, and five between 40 and 52 weeks.

Assessment of residual β -cell function with MMTT

Individual C-peptide responses during the MMTT are shown in Supplementary Table 1. The median C-peptide concentration at baseline was 0.29 nmol/L (interquartiles range 0.26–0.40), and only three had baseline levels <0.2 nmol/L. The median peak stimulated C-peptide concentration was 0.87 nmol/L (interquartile range 0.57–1.11; range 0.12–1.43 [Table 1]), and only one subject (a 17-year-old with T1D of 49 weeks' duration) had a peak level <0.2 nmol/L. C-peptide responsiveness was well maintained throughout the first year of T1D with median peak stimulated C-peptide values that did not differ between subjects in different disease duration bins. In addition, Spearman correlation between peak C-peptide values and duration of diabetes was -0.25 (95% CI -0.61 to 0.21) (Supplementary Fig. 1). Mean plasma glucose levels were 112 ± 29 mg/dL at baseline,

reached values of 216 ± 62 mg/dL at 90 min, and fell to 120 ± 54 mg/dL after 240 min.

Plasma glucagon responses to hypoglycemia in non-T1D and T1D subjects

The median (25th–75th percentile) increases in plasma glucagon in response to hypoglycemia in nondiabetic and T1D subjects are shown in Table 1, and individual responses are shown in Supplementary Table 1. As shown in Table 1, the glucagon response to hypoglycemia was significantly reduced in the children and adolescents with T1D compared with that in the nondiabetic young-adult subjects ($P = 0.02$). Moreover, 7 of the 21 subjects with T1D failed to achieve a glucagon response ≥ 12 pg/mL, and 4 of those subjects had absent responses (peak values <4 pg/mL). In contrast, only 1 of the 12 young-adult control subjects failed to achieve a glucagon response ≥ 12 pg/mL, and the rise in glucagon level was 11 pg/mL. The median incremental glucagon AUC was 1.5-fold higher in the nondiabetic subjects compared with the T1D subjects (Table 1).

The difference in the glucagon response was noted despite achievement of similar nadir glucose levels in the two groups (T1D 51 ± 4 mg/dL vs. nondiabetic control 51 ± 2 mg/dL, P value = 0.78). Supplementary Fig. 2 depicts the change in the glucose and glucagon responses in the nondiabetic and T1D subjects during the hypoglycemic portion of the clamp.

As shown in Fig. 1, all seven of the subjects who had subnormal/absent glucagon responses had peak C-peptide responses that ranged between 0.54 and 1.12 nmol/L and the subject with the lowest C-peptide response during the MMTT

Table 1—Summary of C-peptide during MMTT and glucagon during hypoglycemic clamp

	T1D subjects	Nondiabetic subjects	P*
N	21	12	
MMTT C-peptide (nmol/L)			
Baseline	0.29 (0.26–0.40)		
Peak	0.87 (0.57–1.11)		
Incremental AUC	0.31 (0.28–0.44)		
Hypoglycemic clamp (pg/mL)			
Baseline	34 (25–38)	43 (35–54)	
Peak	50 (37–72)	93 (60–111)	
Rise	18 (7–32)	38 (19–66)	0.02
Incremental glucagon AUC	9.8 (1.5–21.2)	14.8 (8.8–31.1)	0.11

Data are median (interquartile range) unless otherwise indicated. **P* value from Wilcoxon rank sum test.

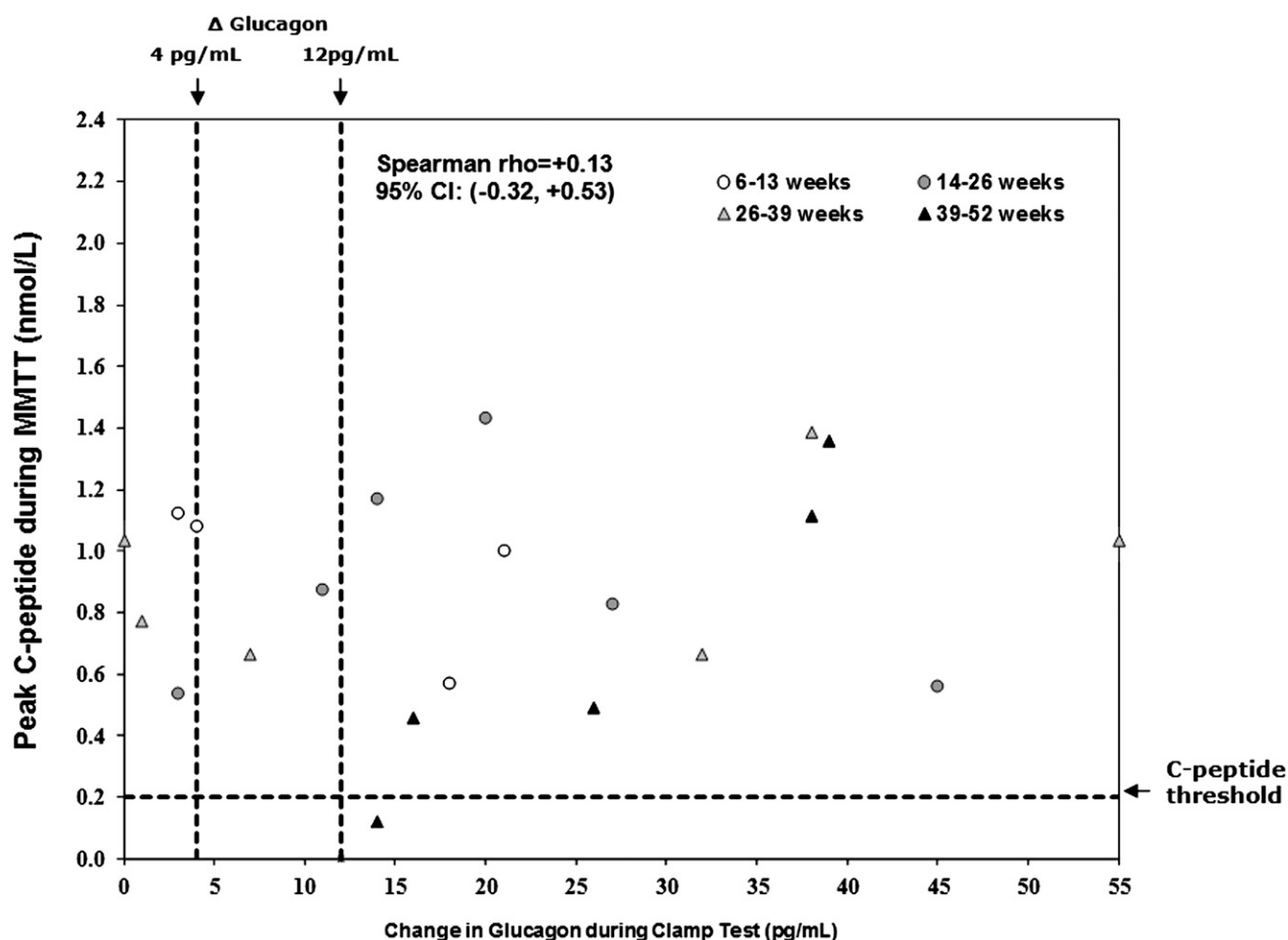


Figure 1—Peak C-peptide vs. change in glucagon for T1D subjects.

had a 14 pg/mL rise in plasma glucagon with hypoglycemia. In the group as a whole, Spearman ρ was 0.13 (95% CI -0.32 to 0.53) for the change in plasma glucagon concentrations with hypoglycemia with the peak C-peptide level and 0.06 (-0.39 to 0.48) for the incremental C-peptide AUC during the MMTT. Furthermore, Spearman ρ was 0.35 (-0.11 to 0.67) (Fig. 2) for the glucagon response to hypoglycemia and duration of diabetes.

CONCLUSIONS—A major aim of this study was to examine the early natural history of the loss of the glucagon responses to hypoglycemia in children and adolescents with T1D, using the gold-standard hypoglycemic insulin clamp procedure. In order to do so, youth with T1D were enrolled in the study, in which the duration of T1D was fairly evenly distributed over the first 12 months of treatment of the disease. While current ethics standards of pediatric clinical research precluded the performance of

hypoglycemic clamps in age-matched healthy control children, we were fortunate to have access to stored glucagon samples that were obtained from 12 healthy young-adult subjects in a different study under nearly identical hypoglycemic clamp conditions at the DirecNet Center located at Washington University.

We used a statistical method based on the coefficient of variation of split duplicate measurements of plasma glucagon levels to derive a minimum threshold (12 pg/mL) that represented a meaningful increase in plasma glucagon concentrations above baseline values. The study results would not have been affected if we had used the minimal rise in plasma glucagon that was observed in the non-diabetic subjects (11 pg/mL) as the threshold.

As we anticipated, approximately one-third of our youngsters had subnormal plasma glucagon responses to hypoglycemia and four of the seven lost glucagon responsiveness altogether. We also expected to see a progressive decline in the

glucagon response over time during the first year of T1D, but our cross-sectional results did not demonstrate such a relationship. These findings are similar to those recently reported by Siafarikas et al., who also found that glucagon responses to hypoglycemia are commonly lost during the first year of diabetes in adolescents with T1D (14).

Based on the intransient hypothesis, we had expected that loss of residual β -cell function, as reflected by a reduction in stimulated C-peptide levels using the gold-standard MMTT, would be associated with a corresponding loss of glucagon responses to hypoglycemia. However, in contrast to the loss of glucagon responses to hypoglycemia, the C-peptide response to the MMTT was surprisingly well preserved during the first year of T1D, with fasting baseline C-peptide levels of ≥ 0.2 nmol/L in all but three subjects and meal-stimulated values ≥ 0.2 nmol/L in all but one subject. Consequently, in the group of diabetic subjects as a whole there was no significant correlation between the

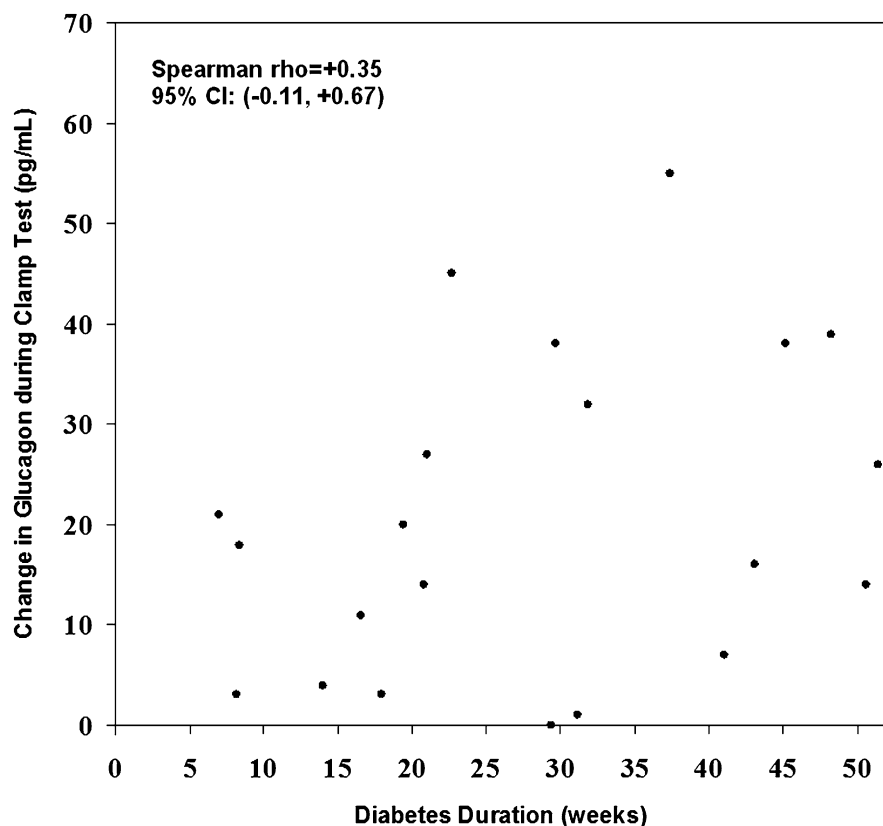


Figure 2—Change in glucagon vs. duration of T1D (N = 21).

magnitude of the increase in plasma glucagon to hypoglycemia and the MMTT-stimulated C-peptide level. Even more important, peak stimulated C-peptide values ranged between 0.54 and 1.12 nmol/L in the four patients who had negligible plasma glucagon responses, whereas the one patient with very low basal and stimulated C-peptide levels had a rise in plasma glucagon that was within the nondiabetic range.

Even though plasma glucagon responses to hypoglycemia were blunted in our subjects, only one subject had a history of a severe hypoglycemic event and continuous glucose monitoring profiles that were obtained in this population prior to the hypoglycemic clamp study demonstrated negligible exposure to biochemical hypoglycemia (27). These data provide further evidence that the retention of residual endogenous insulin secretion plays a key role in mitigating against the risk of severe hypoglycemia during intensive treatment, as first demonstrated in the DCCT study (26). Additionally, as will be reported separately, our subjects also retained vigorous epinephrine responses to hypoglycemia (28). Therefore, despite loss of the glucagon response in a subset of subjects, other counterregulatory

mechanisms protect patients from severe hypoglycemia during the partial remission phase of the disease.

On face value, the results of this study are inconsistent with the inraislelet hypothesis that the loss of glucagon responses to hypoglycemia is closely related to the loss of residual insulin secretion. However, several limitations of the current study caution against making too broad a conclusion based on these data alone. The glucagon assay has changed over the years, making comparison of our results with prior studies quite difficult. In addition, with this assay it has yet to be determined what magnitude of increase in circulating glucagon concentrations is physiologically important. While we used the gold-standard method for inducing hypoglycemia, the continuous insulin infusion itself lowers circulating glucagon levels during euglycemia and that may have resulted in some blunting of glucagon responses to hypoglycemia (29). Conversely, we cannot exclude the possibility that increased glucagon responsiveness might be observed in response to a more severe hypoglycemic stimulus. Although we targeted a glucose level of ~55 mg/dL during the hypoglycemic portion of the clamp, it is notable that

both the T1D and nondiabetic groups achieved a similar mean nadir glucose of 51 mg/dL. Therefore, our subjects, on average, reached the same level of hypoglycemia used by other investigators (14).

Investigators have assessed glucagon response to hypoglycemia while manipulating endogenous insulin secretion to demonstrate the plausibility of the inraislelet insulin hypothesis (17,19–24). However, these studies were completed in nondiabetic individuals (19,20,22,24), subjects with type 2 diabetes (23), or adults who were C-peptide deficient (17,21). Therefore, in the current study of youth with T1D who were not expected to be insulin deficient, we used stimulated C-peptide from an MMTT to estimate residual β -cell function. This measure has become the gold standard to assess residual β -cell function and is often used as an enrollment criteria and outcome measure for therapies aimed at β -cell preservation (30). However, as our comparisons of residual β -cell function and glucagon response to hypoglycemia were conducted using two separate methodologies, it is possible that true assessment of the intricate inraislelet relationship may be limited. Had our data demonstrated a clear relationship between C-peptide and glucagon response to hypoglycemia, it is possible that investigators and clinicians could use the C-peptide levels to estimate whether a positive glucagon response would be expected in a particular subject.

The longitudinal follow-up of these subjects during their second year of T1D is ongoing, and it should provide answers to some remaining questions by evaluating whether the change in C-peptide has any relationship with the change in glucagon responses over time in year 2 compared with year 1. We will also be able to determine whether the patients who had negative glucagon responses during initial testing remained nonresponsive on follow-up testing.

Acknowledgments—This study was supported by National Institutes of Health/National Institute of Child Health and Human Development grants HD-41890-10, HD-41906-10, HD-41908-10, HD-41915, HD-41918, HD-56526, UL1RR-024992, UL1RR-024139, UL1RR-024979, UL1RR-025744, and UL1RR-024150. J.S. received funding from the Pediatric Endocrine/Diabetes Physician Scientists (Grant K12 DK094714-01). Stanford University received funding from JDRF and the Helmsley Foundation for industry-sponsored research. A.M.A. received funding from the

Harold Amos Medical Faculty Development Program (Grant KL2RR024994).

C.K. received payment for consultancy to Medtronic. B.B. received research support for both principal investigator-initiated studies and industry-sponsored studies from Medtronic MiniMed; received payment for consultancy to Sanofi-Aventis, BD Biosciences, Roche, GlySens, Debiotech, Unomedical, Animas, and Bayer; received payment for development of educational presentations from Children with Diabetes; and received travel/accommodations/meeting expenses from the Helmsley Foundation for T1D Exchange. Stanford University received funding from Medtronic MiniMed and DexCom for industry-sponsored research. N.H.W. served on the data-monitoring board and received payment from both Daiichi Sankyo and Novo Nordisk. L.F. received payment serving on the clinical advisory board of Tandem Diabetes Care. S.W. received payment for consultancy to Animas, Novo Nordisk, and BD Biosciences; received payment for lectures including service on speakers bureaus for Eli Lilly; and received payment from stock for InsuLine Medical. No other potential conflicts of interest relevant to this article were reported.

J.S., D.X., K.J.R., and W.V.T. researched data, contributed to discussion, wrote the manuscript, and reviewed and edited the manuscript. R.W.B., C.K., B.B., N.H.W., L.F., E.T., S.W., and A.M.A. researched data, contributed to discussion, and reviewed and edited the manuscript. R.W.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented in abstract form at the 72nd Scientific Sessions of the American Diabetes Association, Philadelphia, Pennsylvania, 8–12 June 2012.

The authors thank all of the subjects and their families for their participation.

APPENDIX—The DirecNet Study Group is as follows (listed in alphabetical order with clinical center name, city, and state). Personnel are listed as PI, principal investigator; I, co-investigator; and C, coordinator.

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