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Evaluation of a sensitive and specific radioimmunoassay for pancreatic glucagon in human plasma and its clinical application

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Summary

A glucagon radioimmunoassay employing antiserum specific for pancreatic glucagon is described. Glucagon was radioiodinated by the chloramine T technique and purified on QAE-Sephadex A 25 to a specific activity of 225 μCi/μg.

The standard curve allowed measurements from 12 to 500 pg/ml with sensitivity of 17.5 pg/ml, precision of 6.3–14.9% (CV, within-assay) and 5.6–10.7% (CV, between-assay). Recovery was between 82 and 112%.

Fasting plasma glucagon levels in diabetics, obese subjects, acromegalics and patients with Cushing's syndrome were greater than in normals (22.0 ± 91 pg/ml; mean ± SD). Very low glucagon levels after oral glucose suppression (15.2 ± 3.1 pg/ml) in normals and greatly increased values after arginine in insulin-dependent diabetics (271.0 ± 132.3 pg/ml) could be determined.

Introduction

Although glucagon was the second hormone to be measured, in 1959, by Unger and co-workers [1] using the radioimmunochemical technique of Yalow and Berson, its measurement has proven considerably more difficult than that of the first one, insulin.

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The major problems were glucagon degradation, weak antigenicity and cross-reaction between pancreatic and gut glucagon. These problems may explain the wide range of glucagon levels reported in the literature.

The present report describes a highly sensitive and specific glucagon radioimmunoassay employing a carefully prepared tracer, with great stability after QAE-Sephadex purification [2], and an antiserum specific for pancreatic glucagon. We also evaluated the operational characteristics of the assay, and measured fasting plasma glucagon levels in several conditions of glucose derangements. Finally, the validity of the assay was confirmed by its application after glucagon stimulation and suppression.

Material and methods

The glucagon radioimmunoassay was developed according to principles described by Alford et al [3] employing twice crystallized porcine glucagon for iodination supplied by NOVO (Lot No. 6501575) and pork glucagon standard supplied by the Medical Research Council (69/194). The antiserum specific for pancreatic glucagon, RCS5 (C terminal reacting) raised in rabbits, was supplied by Dr. S.R. Bloom.

Glucagon was radioiodinated by a modification of the chloramine T technique [2] (10 μg: 0.5 mCi). The monoiodinated glucagon component was purified by anion exchange chromatography in QAE-Sephadex A 25 (Pharmacia, Uppsala, Sweden) at 20°C, according to Jørgensen and Larsen [2].

Glucagon radioimmunoassays were set up with standard curves made up with control 'zero' plasma prepared from pooled, time-expired blood bank plasma treated by repeated freezing and thawing and then incubated at 56°C for 30 min to lower the glucagon content [4]. This plasma was tested in the assay standard curve in comparison with two others, one extracted with 5% charcoal Norit OL (Hopkin and Williams, Chadwell Heath, Essex, UK) and the other prepared by an affinity chromatography technique employing glucagon antibody coupled to sepharose beads [3], supplied by Dr. S.R. Bloom, to eliminate all endogenous glucagon. All these treated plasma samples were stored deepfrozen after adding 0.01% thimerosal. They were thawed immediately prior to use.

The assays were carried out in duplicate, alternating the control 'zero' plasma between groups of unknown samples to verify any change in the tracer reactivity throughout the assay.

A mixture of 0.4 ml assay buffer (0.05 mol/l veronal containing Trasylol and human serum albumin), 0.2 ml of unknown plasma samples or increasing concentrations of standard glucagon (from zero to 348.5 pg) in 0.2 ml of glucagon-free plasma, 0.1 ml of anti-glucagon serum with appropriate dilution (1 : 40 000) and 0.1 ml of [^{125}I]glucagon (10 pg) were incubated for 5 days at 4°C. Afterwards, a mixture of 0.4 ml of charcoal Norit OL (20 mg) coated with dextran T 70 (2 mg) (Pharmacia) and time-expired blood bank plasma (8.4%) suspension in 0.05 mol/l veronal buffer, pH 8.0, was added with constant stirring in a 4°C water-bath. After centrifugation at 2500 rpm for 20 min at 4°C the supernatant was aspirated by suction through a

Pasteur pipette and the radioactivities of the precipitate and the supernatant were measured. The percentage of [^{125}I]glucagon bound to its antiserum was calculated in relation to the total radioactivity.

The specific activity of the tracer used in the assays was determined by the method of self-displacement [5], treating a double amount of tracer as the unknown sample.

The validation of the radioimmunoassay results was analysed using the following parameters: sensitivity, precision, specificity and accuracy.

The sensitivity was evaluated by incubating 18 replicates to determine the percentage binding of tracer to antiserum in the absence of glucagon standard and with the lowest concentrations of the standard (17.5 and 35.0 pg/ml) used in the standard curves. The differences in the percentage bound between the standard concentrations and the zero level were analysed by Duncan's multiple range test [6].

The within-assay reproducibility was evaluated estimating, in the same assay, three pooled human plasmas containing high, medium and low glucagon levels respectively, each with 18 replicates. Between-assay precision was estimated in five separate assays in duplicate. The within- and between-assay precision profiles [7] were obtained using data from human plasmas with different concentrations.

Specificity was verified assaying different dilutions of pooled human plasma samples containing a high endogenous level of glucagon. The dilutions were prepared in glucagon-free plasma, performing five replicates at each dilution level. Linear regression analyses were made between the dilutions and the mean corresponding values and the correlation coefficient was calculated. The displacement of [^{125}I]glucagon by diluted pooled human plasma and porcine pancreatic glucagon standards was also compared.

Accuracy was evaluated by determining the recovery of various concentrations of unlabeled pork glucagon standard added to pooled human plasma of known glucagon concentration (assayed with 8 replicates). Five replicates were made at each level. The linear regression between the expected theoretical and the found values as well as the respective correlation coefficient were determined.

For pancreatic glucagon determination, blood samples were placed promptly after collection in heparinized tubes (10 units/ml) containing Trasylol (4000 kIU/ml), the mixture centrifuged immediately at 4°C and plasma separated and kept frozen at −20°C in plastic tubes until assayed.

Fasting levels of plasma glucagon were measured in 58 individuals of various ages subdivided into six groups consisting of 19 normal non-obese subjects (aged from 21 to 56 years, 14 females and 5 males), 20 diabetics (aged from 17 to 63 years, 9 females and 11 males with fasting blood glucose level ranging from 6.3 to 22.0 mmol/l), 5 obese non-diabetics (aged from 22 to 37 years, all females, with body weight exceeding 160% of ideal body weight — IBW), 5 obese with impaired glucose tolerance (IGT) (aged from 26 to 47 years, all females, with body weight exceeding 140% of IBW, and fasting blood glucose ranging from 3.6 to 5.9 mmol/l), 7 active acromegalics (aged from 20 to 50 years, 3 females and 4 males, with body weight ranging from 122 to 165% of IBW and blood sugar in the normal range in all except two), and finally, two patients with Cushing's syndrome: one female, 34 years old

and one male 25 years old, both with normal weight and fasting blood glucose. The obese individuals were classified according to criteria proposed by the National Diabetes Data Group [8].

Blood glucose was measured with a Technicon Auto-Analyser by the ferricyanide method [9].

Arginine monohydrochloride was infused intravenously at a rate of 0.5 g/kg for 30 min (0–30 min) in 9 normal subjects and 5 insulin-dependent diabetics (IDD) to stimulate glucagon secretion. Blood samples were collected 30 min before and at every 10 min during the first hour (0–60 min) and at 90 min after starting the infusion for glucose and glucagon measurements.

A 75 g oral glucose tolerance test (OGTT) was also carried out in these subjects. Blood samples were withdrawn from the antecubital vein immediately before and at 30, 60, 90, 120 and 180 min after glucose ingestion (15 min) for glucose and glucagon determinations.

All IDD subjects in whom disease duration was longer than 5 years, were kept on NPH insulin (20–170 U daily). No insulin was given at the time of testing.

All studies were carried out after an overnight fast and absolute rest for at least 1 h before testing. Statistical comparisons on all experiments were made by Student's *t*-test for unpaired observations.

Results

Fractions corresponding to the peak of the anion-exchange chromatogram in the [^{125}I]glucagon purification, with binding of the order of 55% after a brief incubation with concentrated antiserum, were pooled and stored at $-20°C$ until use as the tracer in routine radioimmunoassays.

Labeled glucagon was stable for at least three months and had specific activity of about 225 $\mu Ci/\mu g$. Thus the same tracer could be used during this period by modifying the amount of labeled antigen according to the decay time, using a lower specific radioactivity.

Fig. 1 illustrates the displacement of the tracer by glucagon standards prepared in three different glucagon-free plasmas: treated time-expired blood bank plasma, plasma extracted with a specific glucagon immunoadsorbent (glucagon antibody-sepharose beads complex) and one extracted with charcoal. The lower limit of detection in each assay was 13.9, 12.5 and 25.1 pg of glucagon per ml, respectively. We chose treated time-expired blood bank plasma for use in the assays, since it showed a curve parallel to that prepared using plasma extracted with immunoabsorbent, and in addition it was easily obtained.

The usual glucagon radioimmunoassay standard curve allowed the measurement from 12.0 to at least 500 pg/ml of glucagon.

Radioimmunoassay sensitivity was evaluated with 18 replicates comparing, by Duncan's test, the percentage bound between concentrations of two lowest glucagon standards (17.5 and 35 pg/ml) and the plasma free-glucagon (zero level). It was found that the differences between the three mean percentages of bound ligand were highly significant ($p < 0.0001$). Likewise, the percentage bound values for glucagon

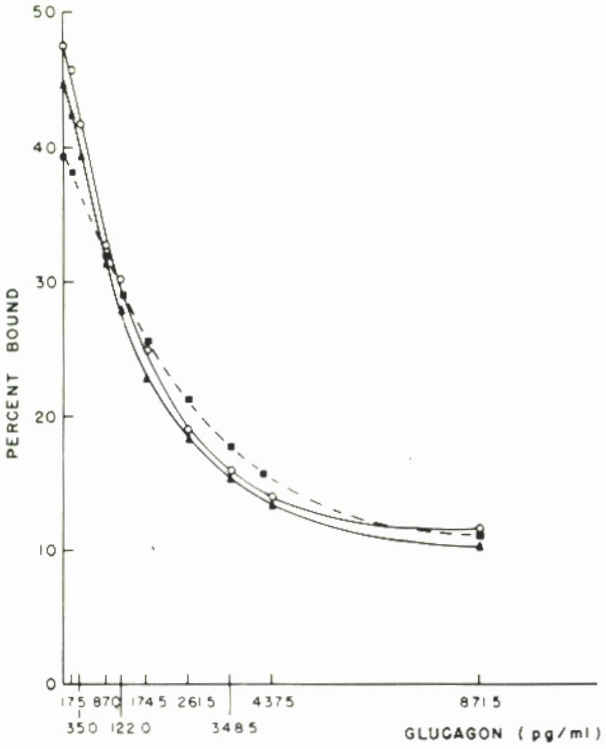

Fig. 1. Comparison of the displacement of [^{125}I]glucagon by glucagon standards in the presence of different glucagon-free plasmas: ▲———▲, time-expired blood bank plasma; ○———○, extracted by immunoabsorbent (glucagon antibody-sepharose beads complex); ■———■, charcoalized plasma.

standards were significantly different from those at the zero level ($p < 0.05$).

Within-day precision studies gave coefficients of variation (CV) of the order of 14.9, 5.1 and 6.3% for plasma samples of low (13.6 pg/ml), medium (65.3 pg/ml) and high (104.2 pg/ml) glucagon mean content, respectively. In the between-assay reproducibility studies, CVs were 10.7, 4.6 and 5.6% for similar plasmas with glucagon mean levels of 14.6, 68.3 and 110.8 pg/ml respectively. Precision profiles indicated that when the limit of CV is fixed to the maximum of 10%, the acceptable range for glucagon assay is 20–114 pg/ml and 16–116 pg/ml for within- and between-assay respectively. For glucagon values in the range of 15–20 pg/ml, however, the intra-assay CV was of the order of 15%.

The specificity study indicated a significant correlation between the dilution volumes of pooled human plasma with a high endogenous glucagon level (151.3 pg/ml) and the found glucagon values. The linear regression equation was $y = 0.7777x - 3.9061$ with a significant correlation coefficient of 0.9999 ($p < 0.001$). Glucagon values corresponded to those of diluted plasma, when the standard curve was made up with porcine glucagon standards.

The recovery of unlabeled porcine glucagon standard added to a pooled human

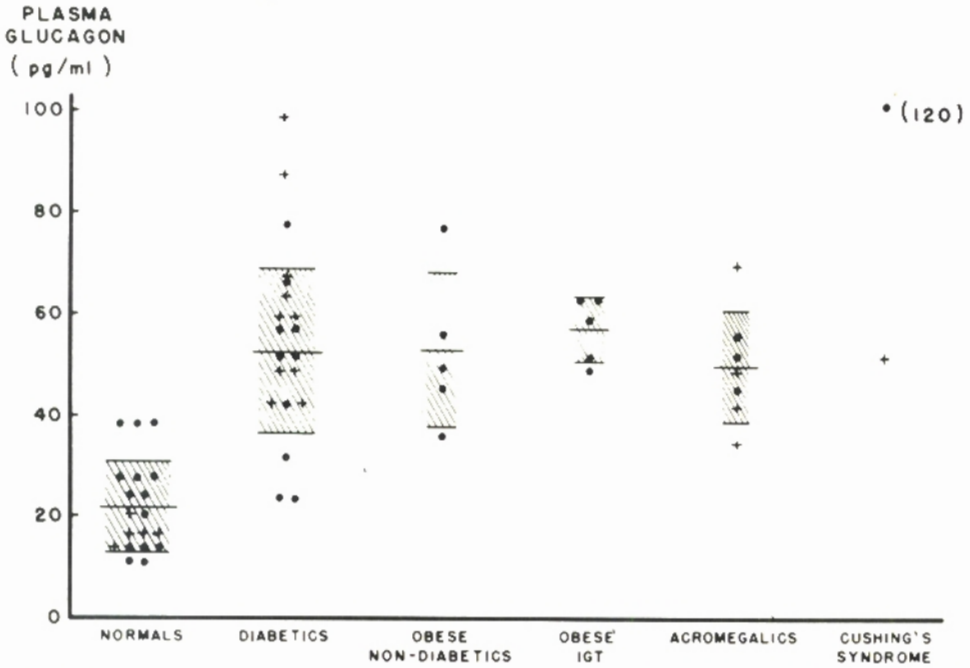

Fig. 2. Fasting plasma pancreatic glucagon concentrations in normal subjects ($n = 19$), diabetics ($n = 20$), obese non-diabetics ($n = 5$), obese IGT ($n = 5$), acromegalics ($n = 7$) and patients with Cushing's syndrome ($n = 2$). Females are indicated by circles (●) and males by crosses (+). Hatched areas represent the mean ± SD for the groups.

plasma (mean ± SD of 22.7 ± 1.9 pg/ml) ranged from 82 to 112% and the correlation between the values of the glucagon standard added and those recovered was significant with a coefficient of 0.9997 ($p < 0.001$). The linear regression equation was $y = 1.485x - 10.1279$.

Fig. 2 illustrates the ranges of glucagon values obtained on overnight-fasting plasma from five study groups. In the normal subject group, the plasma glucagon concentration ranged from 11 to 38 pg/ml with a mean ± SD of 22.0 ± 9.1 pg/ml. In the diabetic group the concentration was 55.2 ± 16.1 pg/ml, ranging from 24 to 97 pg/ml. In the obese non-diabetics it was 53.0 ± 15.0 pg/ml ranging from 38 to 77 pg/ml while in the obese IGT it was 57.2 ± 6.3 pg/ml ranging from 49 to 63 pg/ml. Likewise, in the acromegalic group it was 49.9 ± 11.1 pg/ml, ranging from 35 to 70 pg/ml. Two Cushing's syndrome patients exhibited very high basal glucagon levels. Basal glucagon mean concentrations were significantly higher in all groups of patients compared to normals ($p < 0.001$). In the two cases of Cushing's no statistical analysis was done.

During arginine infusion, the normal subjects (8 females and 1 male), whose ages ranged from 21 to 41 years, exhibited a significant glucagon rise within 10 min to a peak value at 30 min (Fig. 3, a). The mean ± SD peak of glucagon levels was 85.1 ± 44.7 pg/ml and that of blood glucose was 5.3 ± 0.9 mmol/l. Five IDD evaluated (1 female and 4 males) with ages ranging from 18 to 52 years, revealed a

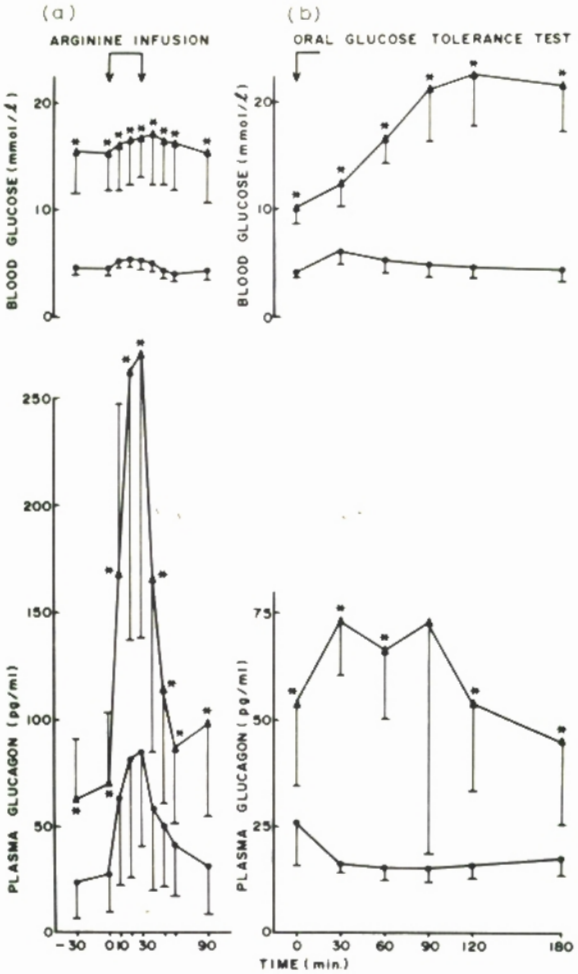

Fig. 3. (a) Blood glucose and plasma glucagon concentrations during an arginine infusion (0.5 g·kg^{-1}·30 min^{-1}) in 9 normal (●———●) and 5 diabetic (▲———▲) subjects. Means ± SD are indicated. Asterisks denote significance of the difference between means of the two groups ($p < 0.05$). (b) Blood glucose and plasma glucagon levels in 9 normal (●———●) and 5 diabetic (▲———▲) subjects during 3 h, 75 g oral glucose tolerance test. Means ± SD are indicated. Asterisks denote significance of the difference between means of the two groups ($p < 0.02$).

significant glucagon rise also reaching its peak at 30 min following the commencement of arginine infusion. Their mean ± SD glucagon peak was 271.0 ± 132.3 pg/ml, while that of blood glucose was 17.1 ± 4.9 mmol/l in the 40-min samples. The mean plasma glucagon and blood glucose levels were significantly higher than the normal subjects at all times of sampling ($p < 0.05$).

Normal subjects presented a progressive decrease in the mean plasma glucagon levels during the first hour after glucose load attaining the nadir values (mean ± SD)

at 60 (15.6 ± 3.2 pg/ml) and 90 min (15.2 ± 3.1 pg/ml), and subsequently showed a small increase to 17.5 ± 4.0 pg/ml in the 180 min sample. The blood glucose levels were maximal at 30 min (6.1 ± 1.2 mmol/l) and decreased progressively to the basal value at 180 min (4.3 ± 0.9 mmol/ml) (Fig. 3, b). On the other hand, the IDD subjects showed an elevation of the mean glucagon levels at 30 min (72.6 ± 11.3 pg/ml) which remained high until 90 min (73.8 ± 55.2 pg/ml) and then decreased to levels below basal at 180 min (45.2 ± 19.8 pg/ml). These levels were significantly higher than those in the normal subjects at all times of sampling ($p < 0.02$) except at 90 min. The IDD also presented mean blood glucose values significantly higher than the normals at all times during OGTT ($p < 0.005$).

Discussion

The glucagon tracer purified by anion exchange chromatography in QAE Sephadex A-25 [2] has a long shelf-life and high specific activity suitable for use in glucagon radioimmunoassays even after 3 months of storage at −20°C, without significant alterations in sensitivity or characteristics of the binding curve.

Regarding the glucagon-free plasma used to dilute the glucagon standards, when extracted by immunoabsorbent, it seemed to facilitate greater binding of tracer to the antiserum and its displacement from antibody by the glucagon standards than the two other plasmas analyzed (Fig. 1). However, standard curves from treated time-expired blood bank plasma presented similar results. Considering that this is easily obtainable and much less expensive, we used the latter for our assays. Our findings are in agreement with those of Alford et al [3]. Therefore, diluting glucagon standards in glucagon-free plasma and employing 20 mg of charcoal for the separation of free from bound hormone, the tracer competed in a consistent and equal manner with unlabeled glucagon, producing standard curves which allowed the measurement of glucagon levels as low as 12.0 pg/ml with acceptable precision. Moreover, the use of this treated plasma as a control 'zero' plasma between groups of unknown samples in each run, gave an indication of any drift occurring in the tracer, in the absence of unlabeled hormone, thus suggesting the presence of interfering factor(s) in the assay.

The glucagon radioimmunoassay described herein was studied with respect to sensitivity, precision, specificity and accuracy. Its high sensitivity and the useful wide range of standard curves for plasma determinations (from 12 to approximately 500 pg of glucagon per ml), demonstrate the applicability of the method in clinical work, for example in tests involving stimulation and depression of pancreatic glucagon secretion (Fig. 3).

Analyzing our within- and between-assay precision profiles, the corresponding working ranges were about the same when the CV was lower than 10%, allowing measurement of all (low/high) glucagon levels. While the CV for a high glucagon level corresponding to approximately 100 pg/ml was 6.3% ($n = 18$) and 5.6% ($n = 5$) for within- and between-assay respectively, in the same evaluation reported by Alford et al [3], with a similar method, for a glucagon concentration of 90 pg/ml, it

was 7.8% ($n = 22$) for intra- and 14.9% ($n = 9$) for inter-assay precision, respectively.

The linearity and high correlation found between the plasma dilutions and the measured glucagon concentrations is highly suggestive of the specificity of the assay. The close correlation between several dilutions of plasma containing various amounts of glucagon within the standard curve indicates that plasma samples and standards behave similarly in the assay system.

The antiserum used (RCS5), as demonstrated by Alford et al [3], is pancreatic glucagon-specific and does not cross-react with gut glucagon also present in plasma.

The recovery of added glucagon ranging from 82 to 112%, with a mean value close to 100%, and a correlation coefficient exceeding 0.98 at different levels of added glucagon standard, indicates a high degree of accuracy of the method for a range of low to very high glucagon levels.

Fasting levels of plasma pancreatic glucagon in humans, previously reported in the literature, show a wide range of variation probably due to methodological differences, particularly in the use of non-specific antisera. The fasting pancreatic glucagon levels presented here are much lower than those reported from other laboratories [10] including Unger's (75 ± 4 pg/ml = mean $\pm$ SEM) [11] but are similar to those obtained by Alford et al [3] using the same RCS5 antiserum.

We have observed significantly greater basal pancreatic glucagon levels (11.8 ± 4.5 mmol/l) in diabetics with fasting hyperglycemia, all of them non-obese with an ideal body weight of 104.3 kg $\pm$ 9.8% (mean $\pm$ SD) than in the control subjects, as also reported by other investigators [3,10]. This suggests reduced suppressibility of the alpha-cell to hyperglycemia in diabetes.

The finding of high basal glucagon levels in obese subjects, with and without impaired glucose tolerance, a finding not observed by all investigators [12], is thought to be related to the insensitivity of alpha-cell to insulin action in the obese subjects. It has been postulated that the alpha-cell participates in whole body insulin resistance [13].

By the same token, acromegalics, all of whom had normal or slightly increased fasting blood glucose and greatly elevated basal insulin levels (unpublished data), presented correspondingly elevated fasting plasma glucagon levels, as previously described by Seino et al [14] who also found an excessive response to arginine. This, as well as our findings, in Cushing's disease, are examples of insensitivity of the alpha-cell in these well known conditions of insulin resistance.

As a further application of our method in evaluating its reliability in the measurement of high glucagon levels after stimulation and of low levels after its suppression, we performed arginine infusion and oral glucose tolerance tests. As expected, after arginine, the pancreatic glucagon secretion was uniformly stimulated in normals and markedly exaggerated in IDD despite their hyperglycemia, in accordance to previous reports [15,16].

The results of the OGTT agree with the findings of Heding et al [10] showing a decrease in the glucagon levels caused by hyperglycemia in the normal subjects and a lack of glucagon suppressibility in IDD.

The high sensitivity, therefore, of the pancreatic glucagon radioimmunoassay as well as its precision allowed us the measurement of very low glucagon levels

suppressed by the OGTT in normals and of high levels stimulated by the arginine infusion in IDD.

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References

1 Unger RH, Eisentraut AM, McCall MS, Keller S, Lanz HC, Madison LL. Glucagon antibodies and their use for immunoassay of glucagon. Proc Soc Exp Biol Med 1959; 102: 621–623.
2 Jørgensen KH, Larsen UD. Purification of [^{125}I]glucagon by anion exchange chromatography. Horm Metab Res 1972; 4: 223–224.
3 Alford FP, Bloom SR, Nabarro DN. Glucagon levels in normal and diabetic subjects: Use of a specific immunoabsorbent for glucagon radioimmunoassay. Diabetologia 1977; 13: 1–6.
4 Ohneda A, Yamagata S. Evidence for the presence of inhibitor and some trials for its prevention in radioimmunoassay of plasma glucagon by two-antibody system. Tohoku J Exp Med 1971; 103: 257–268.
5 Morris BJ. Specific radioactivity of radioimmunoassay tracer determined by self-displacement: A re-evaluation. Clin Chim Acta 1976; 73: 213–216.
6 Winer BJ. Statistical principles in experimental designs. New York: McGraw Hill, 1971.
7 Jeffcoate SL. The use of quality control within a laboratory. In: Radioimmunoassay and related procedures in medicine 1977: Proceedings of Symposium, 1977, Berlin. Vienna: International Atomic Energy Agency, 1978, Vol. 2: 57–59.
8 National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 1979; 28: 1039–1057.
9 Hoffman WS. A rapid photoelectric method for the determination of glucose in urine and blood. J Biol Chem 1937; 120: 51–55.
10 Heding LG, Rasmussen SM. Determination of pancreatic and gut glucagon-like immunoreactivity (GLI) in normal and diabetic subjects. Diabetologia 1972; 8: 408–411.
11 Faloona RG, Unger RH. Glucagon. In: Jaffe BM, Behrman HR, eds. Methods of hormone radioimmunoassay. New York: Academic Press, 1974: 317–330.
12 Gossain VV, Mature ML, Kalkhoff RK. Relative influence of obesity and diabetes on plasma alpha-cell glucagon. J Clin Endocrinol Metab 1974; 38: 238–243.
13 Borghi VC, Wajchenberg BL, Cesar FP. Plasma glucagon suppressibility after oral glucose and its relation to insulin secretion in obese non-diabetics and with impaired glucose tolerance. Metabolism 1983, submitted for publication.
14 Seino Y, Taminato T, Gotoy et al. Growth hormone modulation of arginine-induced glucagon release: Studies of isolated growth hormone deficiency and acromegaly. Clin Endocrinol 1978; 9: 563–570.
15 Unger RH, Aguilar-Parada E, Müller WA, Eisentraut AM. Studies of pancreatic alpha cell function in normal and diabetic subjects. J Clin Invest 1970; 49: 837–848.
16 Asplin CM, Paquette TL, Palmer JP. In vivo inhibition of glucagon secretion by paracrine beta cell activity in man. J Clin Invest 1981; 68: 314–318.