The Glycogenolytic Activity of Immunoreactive Pancreatic Glucagon in Plasma

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ABSTRACT Conclusions concerning the physiologic role of pancreatic glucagon in health and its contribution to disorders of carbohydrate metabolism, such as diabetes mellitus, are based entirely on measurements of plasma glucagon by radioimmunoassay. The changes in plasma immunoreactive glucagon can have the metabolic and clinical significance which has been implied, only if the glucagon detected by immunoassay has biological activity. The present study was designed to determine if a relationship between the immunoassayable glucagon and glycogenolytic activity of plasma could be demonstrated.

Plasma specimens obtained from normal and diabetic subjects under widely varying circumstances of alpha cell activity were extracted by a modification of the Kenny technique and the recovery of immunoreactive glucagon was calculated. Glycogenolytic activity of each extract was determined by perfusion in the Mortimore rat liver system, modified so as to detect as little as 1 ng of crystalline glucagon.

A significant correlation between the calculated quantity of immunoreactive glucagon and the glycogenolytic activity of plasma extracts was observed for both normal and diabetic subjects. Most of the glycogenolytic activity was abolished by incubating the extract with antiglucagon serum.

It was concluded that the glycogenolytic activity of extractable glucagon is proportional to its immunoreactivity as calculated from its original concentration in plasma. This would tend to support the view that all or most of the immunoreactive glucagon of plasma is biologically active.

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INTRODUCTION

Recent conclusions concerning the physiologic role of glucagon and its possible contribution to disorders of carbohydrate metabolism are based entirely on measurement of plasma glucagon by radioimmunoassay (1, 2). The observed changes in plasma glucagon can have the metabolic and clinical significance which has been implied only if the glucagon detected by radioimmunoassay is biologically active. There is evidence that the biological activity of glucagon may be destroyed by minor modifications of its structure (3), whereas its immunoreactivity may survive even relatively major structural change, including limited tryptic digestion (4). It seemed possible, therefore, that part or all of the immunoreactive glucagon measured in plasma might be biologically inactive. This study was designed to evaluate the physiologic and clinical significance of changes in glucagon, measured immunochemically in the plasma of normal and diabetic patients, by determining if a correlation exists between the level of glucagon immunoreactivity and glycogenolytic activity in extracts of their plasma.

METHODS

The rat liver perfusion system used by Mortimore (5) was modified slightly. The perfusate consisted of an 18–20% (by volume) suspension of outdated human red cells, washed twice in 0.9% NaCl and twice in Krebs-Ringer phosphate buffer and then suspended in Krebs-Ringer phosphate buffer with 3% (by weight) bovine albumin (Cohn Fraction V) at pH 7.4.

Fed male Sprague-Dawley rats weighing between 100 and 200 g were employed for the liver perfusion. A perfusion apparatus (constructed by Mr. B. Moore, Vanderbilt University School of Medicine, Nashville, Tenn.) with a Harvard peristaltic pump (Harvard Apparatus Co., Inc., Millis, Mass.) was used. The perfusion was conducted at a rate of 7–7.2 ml/min. 1 ml portions of perfusate were withdrawn for glucose analysis from the tubing emerging from the reservoir at 10 min intervals throughout the perfusion.

The sensitivity of the assay was increased by reducing the volume of the perfusate to 35 ml, and prolonging the equili-

bration period from 30 to 60 min. After an hour of perfusion spontaneous glycogenolysis is minimal or absent, which facilitates the detection of small increases in net-hepatic glucose output.

Samples to be tested were injected into the drum reservoir, where they were mixed before reaching the liver. At the end of each experiment, 10 or 20 ng of crystalline glucagon were injected to assess hepatic responsiveness; if no response occurred, the experiment was discarded.

The glycogenolytic activity was taken to represent the net glucose produced from 60 to 100 min of perfusion, expressed as micromoles of glucose per 100 grams of rat body weight. Corrections were made for the amount of glucose present in the portions previously withdrawn. Glucose concentration was determined by the Hoffman ferricyanide method (6) using the Technicon AutoAnalyzer (Technicon Corp., Ardsley, N. Y.).

Extracts of human plasma were prepared by the alcohol precipitation method of Kenny (7), except that the alcohol extract was not precipitated with ether, but was concentrated by flash evaporation in a siliconized flask. The residue was suspended in water, dialyzed overnight against 10^{-2} M Na_2CO_3 at pH 10, and centrifuged at 2000 rpm for 15 min. The supernatant was lyophilized to dryness. The samples were reconstituted with 2–3 ml of 0.2 M glycine in 0.25% human albumin. The reconstituted extracts were added to the perfusion system directly, or in the case of the neutralization experiments, after incubation with 1 ml of anti-glucagon serum or nonimmune serum for 4 days at 4°C. The per cent recovery was determined by the addition of a trace amount of glucagon-^{125}I to the plasma sample before extraction. The recoveries ranged from 24-48%. The amount of immunoassayable glucagon in the extract was estimated by multiplying the original plasma concentration by the volume of plasma extracted and the per cent recovery. Immunoassay of the final extracts was not performed in order to conserve the entire extract for the perfusion experiment, particularly since previous studies in this laboratory had indicated a close correlation between the extraction of glucagon-^{131}I and plasma glucagon.[1]

RESULTS

Validation of sensitive assay of glycogenolytic activity. To determine if the modified liver-perfusion system (5) could detect the glycogenolytic activity of the low quantities of glucagon present in extracts of plasma, its response to 2.5 and 1.0 ng of crystalline glucagon and to a glucagon-free buffer control was determined. In 10 control experiments in which the glucagon-free buffer was perfused, glucose output ranged from 0 to 37.8 μmole/100 g of rat weight and averaged 10.8 μmole/100 g (SEM ±4.4). By contrast, the 2.5 ng dose of glucagon, which gives a perfusate concentration of 100 pg/ml, elicited in every one of nine experiments a brisk rise in glucose production, which ranged from 59.9 to 150.6 μmole of glucose/100 g (SEM ±10.1); this was significantly greater than the response to the buffer control ($P < 0.001$). The perfusion of 1.0 ng of crystalling glucagon, which gives a perfusate concentration of only 40 pg/ml, elicited in six of eight experiments a glycogenolytic response which was higher than 37.8 μmole/100 g, the maximum glucose output obtained with the buffer control. The glucose output ranged from 19.3 to 106 μmole/100 g and averaged 52.9 (SEM ±8.9), which was significantly higher than that of the buffer control ($P < 0.01$) and significantly lower than the response to 2.5 ng of glucagon ($P < 0.01$). A highly significant correlation ($r = 0.82$; $P < 0.001$) between immunoreactivity and glycogenolytic activity was observed in this group of experiments.

Relationship between calculated immunoreactivity and glycogenolytic activity of extracts of normal plasma. To determine if a relationship between the immunoreactivity and glycogenolytic activity of the glucagon in plasma extracts of nondiabetic subjects could be demonstrated, blood samples were obtained from 10 normal volunteers under circumstances of varying alpha cell activity. These circumstances included alpha cell stimulation by arginine infusion, during which plasma glucagon concentration is maximal, averaging more than 300 pg/ml (1), alpha cell suppression by glucose infusion (1), during which plasma glucagon concentration is minimal, averaging less than 60 pg/ml, and alpha cell activity after an overnight fast, when it averages about 110 pg/ml (1).

The relationship of the calculated immunoreactivity of these extracts to their glycogenolytic activity is indicated in Table I. The immunoreactivity of two extracts from plasma obtained during hyperglycemic suppression of glucagon secretion, was calculated to be less than 1 ng, and their glycogenolytic activity was within the range of the buffer control. Extracts of plasma obtained from three fasting subjects and five arginine-stimulated subjects all contained more than 1 ng of glucagon and all gave a glycogenolytic response greater than the highest response observed with the buffer control.

As indicated in Fig. 1, the correlation between calculated glucagon immunoreactivity and glycogenolytic activity in the 10 experiments was slightly significant ($r = 0.67$; $P < 0.05$). The slope of the regression line was somewhat steeper than that obtained with crystalline glucagon; however, the difference was not statistically significant.

To determine if the glycogenolytic activity of plasma extracts from normal subjects was a consequence of its pancreatic glucagon content, rather than of other glycogenolytic substances, the ability of a rabbit antiserum, highly specific for pancreatic glucagon, to neutralize it was tested. 1 ml of the antiserum had been found capable of neutralizing the glycogenolytic activity of more than 2.5 ng of crystalline glucagon. Identical portions of two of the plasma extracts were incubated at 4°C for 4 days either with undiluted specific rabbit antiglucagon serum, or as a control, with undiluted nonimmune serum, after

[1] Unpublished observations.

TABLE I

Glycogenolytic Activity of Plasma Extracts of Normal Subjects

Plasma obtained	Case	Plasma immunoreactive glucagon	Immunoreactive glucagon in extract	Glycogenolytic activity
		pg/ml	*ng*	*μmoles of glucose/ 100 g of rat weight*
During glucose infusion	1	70	0.56	11.2
	2	50	0.25	24.8
After overnight fast	3	90	1.1	59.9
	4	100	1.4	254.5
	5	80	1.1	181.5
During arginine infusion	6	310	2.8	307.8
	7	200	3.0	158.4
	8	590	3.7	257.2
	9	590	2.2	134.5
	10	170	2.8	136.7

which the glycogenolytic activity of each mixture was determined. As shown in Table II (patients 1 and 2), a marked reduction in the glycogenolytic activity of the extracts incubated with antiserum was observed, whereas, the control portion incubated with nonimmune serum, gave full glycogenolytic activity. However, in neither of these two extracts was the glycogenolytic response reduced to within the range of glucagon-free buffer controls, i.e., to 38 μmole of glucose/100 g or less. This incomplete suppression of glycogenolytic activity is compatible with the presence in these plasma extracts of a glycogenolytic factor in addition to and other than pancreatic glucagon, as it seems unlikely that the quantity of glucagon in either extract exceeded the binding capacity of the antiserum employed. Unfortunately, the scarcity of neutralizing antiserum precluded additional experiments.

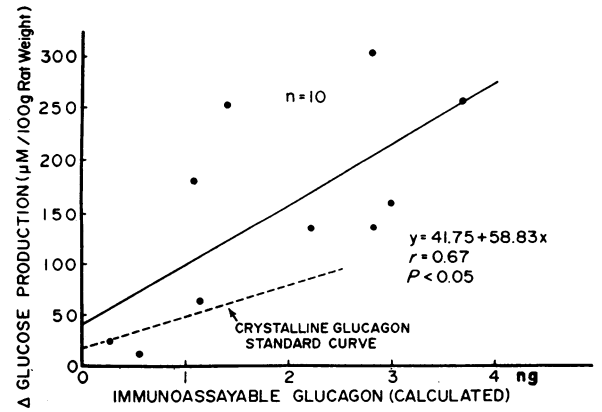

FIGURE 1 The relationship of calculated immunoassayable glucagon to glycogenolytic activity in extracts of plasma obtained from normal subjects after an overnight fast and during infusion of glucose or arginine.

It was concluded that immunoreactive pancreatic glucagon in extracts of plasma has glycogenolytic activity, which is proportional to its immunometrically determined dose, and that the greatest part of this activity can be neutralized with specific antiserum against pancreatic glucagon.

Relationship between calculated immunoreactivity and glycogenolytic activity of extracts of diabetic plasma. To determine if a similar relationship between the immunoreactivity and glycogenolytic activity of the glucagon is demonstrable in the plasma extracts of diabetics, blood samples were obtained from eight diabetic patients after an overnight fast, when plasma glucagon levels are usually in the nondiabetic range, averaging about 118 pg/ml (1), during stimulation of alpha cell secretion by means of an arginine infusion, when plasma glucagon averages more than 450 pg/ml, significantly greater than nondiabetics (1), and in severe diabetic ketoacidosis, when glucagon averages over 400 pg/ml (1).

Table III indicates the calculated glucagon immunoreactivity and glycogenolytic activity of each of the eight extracts. Two of the extracts, from cases 3 and 4, were selected because of the unusually low glucagon concentration of the plasma; the extracts of these plasma specimens contained less than 1 ng of glucagon immunoreactivity, and gave a glycogenolytic response below 38 μmole/100 g, or within the range of response to the buffer control. The other six extracts, all of which contained more than 1 ng of glucagon immunoreactivity, gave a glycogenolytic response well above the range of the buffer controls. These included two plasma extracts from fasting diabetics, two from arginine stimulated diabetics, and two from patients with untreated diabetic ketoacidosis. As depicted in Fig. 2, the correlation be-

TABLE II

Glycogenolytic Activity of Plasma Extracts after Incubation with Antiserum Against Pancreatic Glucagon

Patient	Calculated immunoreactive glucagon	Glycogenolytic activity	
		Extract + antiserum	Extract + nonimmune serum
	ng	*μmoles glucose/100 g rat weight*	
1*	2	53.8	134.5
2*	3.7	78.9	257.3
3**	3.5	18.5	185.3

* Nondiabetic patient.

** Patient in diabetic ketoacidosis.

tween calculated immunoreactivity and glycogenolytic activity was highly significant ($r = 0.85$; $P < 0.01$). As in the case of the nondiabetics, the slope of the regression line was steeper than that obtained with crystalline glucagon, but the difference was not statistically significant.

Table II indicates that the glycogenolytic activity of an extract of diabetic plasma obtained from a patient (patient 3) in severe ketoacidosis, and calculated to contain 3.5 ng of glucagon, was completely suppressed by highly specific antiserum against pancreatic glucagon.

The results indicate a statistically significant relationship between the calculated dose of immunoreactivity and the glycogenolytic activity of the pancreatic glucagon present in extracts of plasma obtained from diabetic patients. In the only such experiment conducted, this immunoreactivity was completely neutralized by a highly specific antiserum for pancreatic glucagon.

DISCUSSION

The foregoing results reveal a statistically significant relationship between the dose of glucagon, as calculated from its radioimmunometric concentration in plasma, and the glycogenolytic activity of plasma extracts obtained from nondiabetic and diabetic subjects under a variety of physiologic and pathophysiologic circumstances. This provides the first evidence in support of the assumption that plasma measurements of glucagon obtained by radioimmunoassay bear a relationship to the level of biologically active hormone. This assumption is open to question in the case of any peptide hormone, but particularly in the case of glucagon, inasmuch as relatively minor modifications of its molecular structure give rise to biologically inactive but immunologically active derivatives (3, 4), and because "large glucagon immunoreactivity" or "LGI", a molecule devoid of glycogenolytic activity, yet immunologically indistinguishable from glucagon (4), has been reported to be present in plasma (8).

Although this study favors the biological activity of immunoreactive plasma glucagon, it falls short of final proof. For technical reasons plasma extracts, rather than samples of whole plasma, were employed in this study, and direct correlation between the glucagon concentration of plasma and glycogenolytic activity was, therefore, not possible. The variability of the recovery of glucagon with this extraction procedure made it necessary to relate the biologic acivity of each extract to its calculated

TABLE III

Glycogenolytic Activity of Plasma Extracts of Diabetic Subjects

Plasma obtained	Case	Plasma immunoreactive glucagon	Immunoreactive glucagon in extract	Glycogenolytic activity
		pg/ml	*ng*	*μmoles glucose/100 g rat weight*
After overnight fast	1	190	3.2	319.4
	2	160	1.5	178.6
	3	30	0.2	30.2
	4	60	0.5	29.7
During arginine infusion	5	700	3.3	279.1
	6	700	2.9	157.4
In ketoacidosis	7	200	1.7	78.0
	8	1500	3.5	185.3

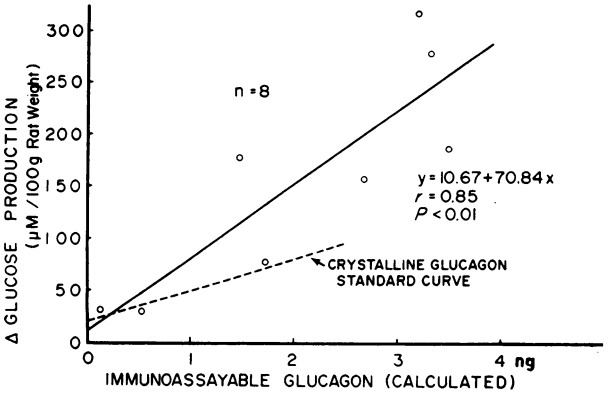

FIGURE 2 The relationship of calculated immunoassayable glucagon to glycogenolytic activity of extracts of plasma obtained from diabetic subjects after an overnight fast and during an arginine infusion.

radioimmunometric content of glucagon, rather than to the initial plasma glucagon level. One can, therefore, legitimately argue that, in the course of the extraction procedure, biologically inactive immunoreactive moieties, originally present in the plasma, were removed either by alcohol precipitation or by dialysis of the extract before the perfusion, and that only intact glucagon remained in the final extract. However, it would be surprising if precipitation with alcohol removed an important fraction of plasma glucagon immunoreactivity, there being no evidence of binding of glucagon to plasma proteins, and the possibility that small immunoreactive derivatives of glucagon were removed by dialysis of the extract is also unlikely. Furthermore, since in these studies, the quantity of extracted immunoreactive glucagon perfused was calculated from the initial plasma content of immunoassayable glucagon multiplied by the recovery of glucagon-^{125}I added to the plasma immediately before the extraction procedure, a sizeable loss of biologically inactive immunoreactivity during dialysis would have resulted in a spuriously high figure for immunoreactivity relative to the quantity of intact glucagon actually present, and the regression slope would have been shallower than that of crystalline glucagon; in fact, however, the slope for plasma was if anything steeper than that for crystalline glucagon, which mitigates against this possibility.

These data, then, provide evidence in favor of the biological activity of circulating immunoreactive pancreatic glucagon, at least in so far as glycogenolytic activity is concerned. Since the gluconeogenic action of glucagon, like its glycogenolytic effect, is believed to be mediated by increased intrahepatic levels of cyclic AMP resulting from stimulation of adenyl cyclase (9), it may be assumed that plasma glucagon has gluconeogenic as well as glycogenolytic activity.

A constant state of relative hyperglucagonemia as determined by radioimmunoassay of plasma, has been reported to exist in diabetic patients (1, 2). The correlation between the calculated quantity of immunoreactive glucagon and the glycogenolytic activity of extracts of plasma from diabetic patients tends to support the notion based on immunometric data alone, that the absence of normal suppression of glucagon secretion by hyperglycemia must be metabolically detrimental and, by opposing the hepatic action of insulin, plays a role in maintaining hepatic glucose production at a higher level than would otherwise prevail. In patients with severe diabetic ketoacidosis, high glucagon values (1, 10) may oppose the hepatic and, perhaps, the antilipolytic actions of insulin; the amount of insulin required to reduce glucose and free fatty acid production in this setting may thus exceed the amount that would be effective in the absence of hyperglucagonemia. The demonstration of glycogenolytic activity in the plasma of hyperglycemic diabetic patients may well have other important pathophysiologic and therapeutic implications in the management of the disease.

The refinement of the Mortimore liver perfusion system employed in this study permits the detection of as little as 40 pg/ml of glucagon, which approaches the sensitivity of most radioimmunoassays for glucagon. This level of sensitivity has been achieved by other groups as well (11, 12). However, this study is believed to be the first in which the pancreatic glucagon of plasma was detected by bioassay and the specificity of the results established by suppression of bioactivity with a specific antiserum. This technique, which can, undoubtedly, be further improved, should be of value in studies of carbohydrate metabolism.

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