Plasma glucagon in diabetes of haemochromatosis: too low or too high?¹

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SUMMARY Glucagon secretion before and during arginine infusions was tested in 11 patients with diabetes associated with haemochromatosis. The results were compared with those obtained in six normal subjects and five patients with haemochromatosis but normal glucose tolerance. The patients with haemochromatosis, regardless of glucose tolerance, exhibited higher levels of plasma immunoreactivity for glucagon (antiserum 30-K) suggesting hyperglucagonaemia. However, additional analysis revealed that a considerable amount of this glucagon immunoreactivity was due to cross-reacting material of high molecular weight, the levels of which were significantly higher in patients with idiopathic haemochromatosis. When this was deducted from the total immunoreactivity measured, the resulting values for true glucagon concentrations were similar to those of normal subjects. The data suggest that (1) patients with idiopathic haemochromatosis, whether or not associated with diabetes, exhibit plasma glucagon levels comparable with those of normal subjects; (2) the plasma of the same patients contains significantly more high-molecular-weight substances reacting with glucagon antiserum 30-K than is present in plasma of normal subjects; and (3) 'hyperglucagonaemia' may be erroneously suggested when glucagon is measured with certain antisera reputed to be specific for glucagon.

Diabetes mellitus associated with idiopathic haemochromatosis has, in the past, often been attributed to a destruction of the pancreatic islets by interstitial reaction to iron deposits (Marble and Bailey, 1951; Peterson, 1960; Bothwell and Finch, 1962). If this were the sole pathogenetic mechanism, the secretion of glucagon should also be deficient and low plasma levels of glucagon would be expected. On the other hand, cryptogenic diabetes mellitus is characteristically associated with raised plasma levels of glucagon (Unger *et al.*, 1970; Muller *et al.*, 1970; Buchanan and McCarroll, 1972; Day and Anderson, 1973) and, by analogy, this would argue in favour of raised glucagon levels in patients with haemochromatosis and diabetes. The recent report by Passa *et al.* (1977) discards the first hypothesis by showing that glucagon secretion is not diminished

in eight patients with diabetes and haemochromatosis. In fact, these authors have reported increased levels of plasma glucagon as measured with the glucagon antiserum 30-K.

In order to dissociate the relative influence of idiopathic haemochromatosis on glucagon secretion from that of diabetes mellitus in this syndrome, we studied 11 patients, exhibiting both diabetes and haemochromatosis, and compared them with five patients having idiopathic haemochromatosis but normal glucose tolerance and with a non-diabetic control group without haemochromatosis matched for age and sex. Furthermore, analysis for high molecular weight plasma moieties known to cross-react with the glucagon antiserum 30-K (Valverde *et al.*, 1974; Weir *et al.*, 1975; Jaspan and Rubenstein, 1977) has permitted calculation of the 'net' true glucagon concentration of a given plasma sample.

Methods

PATIENTS

Sixteen patients with idiopathic haemochromatosis.

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were investigated and compared with a control group of six normal subjects. The patients were outpatients or hospitalised in either the Policlinique Médicale Universitaire in Lausanne, Switzerland, or in the Department of Medicine of the University of Düsseldorf, Germany. The diagnosis of haemochromatosis had been established by typical hyperpigmentation, raised serum iron levels (above 1.7 mg/l) and a saturation of total iron binding capacity exceeding 75% at the time of diagnosis one to 29 years before the study. In all cases, a liver biopsy had been performed, showing on histological examination cirrhosis in 14 and fibrosis in two patients with grade IV iron deposition (Williams, 1968; Dymock *et al.*, 1972). All patients were treated by periodic bleeding. The control subjects were matched by age and sex and reported to the hospital for diseases unrelated to diabetes or other metabolic or gastrointestinal illnesses.

The patients with haemochromatosis were divided into three groups according to their carbohydrate tolerance as assessed by oral glucose tolerance tests (Report of WHO Expert Committee, 1965; Association Suisse du Diabète, 1971): the first group consisted of five patients with a normal tolerance to glucose; the second group comprised seven patients with a pathological tolerance to glucose and included one patient with overt though mild diabetes; the third group included four patients with diabetes requiring insulin treatment. The clinical characteristics of the patients are given in Table 1.

INFUSIONS OF ARGININE

These were performed after an overnight fast, patients lying in a supine position. Arginine HCl (pH 6.4) was infused at a rate of 0.45 g/kg over 40 minutes in 500 ml. At each sampling time, 6 ml of blood was withdrawn from the contralateral antecubital vein into a chilled tube containing 12 mg EDTA (Na₂), and 3000 KIU Trasylol (Bayer AG, Wuppertal, Germany). After centrifugation of the tubes, the plasma was separated and frozen for storage awaiting measurements of glucose, insulin, and glucagon.

ASSAY PROCEDURES

Glucose was measured by the glucose-oxidase method (Bergmeyer and Bernt, 1970) (glucose oxidase was provided by Professor F. H. Schmidt, the Boehringer Company, Mannheim, Germany). Iron was determined according to Lauber (1965) and transferrin by nephelometry (Eckman *et al.*, 1970). Insulin and glucagon were determined by immunoassay using charcoal separation (Herbert *et al.*, 1965; Faloona and Unger, 1974). Insulin antiserum was a gift from Dr P. Wright, Indianapolis, Indiana; glucagon antiserum 30-K was donated by Dr R. H. Unger, Dallas, Texas. In order to assess the contribution of the large-molecular type glucagon immunoreactivity, the plasma samples were divided into two parts: one part was diluted 1:2 with assay buffer (0.2 M glycine, pH 8.8, containing 0.25% human albumin) and the other part was treated v/v by the routine assay charcoal-dextran mixture (1 g charcoal, 0.5 g dextran in 200 ml glycine 0.2 M, pH 8.8) and centrifuged. The supernatant and the diluted serums were assayed for IRG in duplicate in a routine assay using 0.2 ml sample in 1.2 ml total volume (Faloona and Unger, 1974). In order to verify the efficiency of charcoal extraction, samples were applied to Biogel P-30 column chromatography as described by Valverde *et al.* (1974). The columns measured 80 × 1 cm, the elution buffer was glycine 0.2 M, pH 8.8, containing 0.25% human serum albumin and 500 KIU Trasylol per ml. For calibration of the columns, glucagon-¹²⁵I and insulin-¹²⁵I were used. The elution profile of IRG confirmed that charcoal was binding only low molecular weight immunoreactivity (Fig. 1), as described by Weir *et al.* (1975).

The results were analysed statistically using the *t* test as described by Snedecor and Cochran (1967). Results are all expressed as mean ± SEM.

Results

The plasma concentrations of glucose, insulin, and glucagon, before, during, and after arginine administration are shown in Table 2. As expected, plasma glucose was raised and insulin levels lowered in the patients with glucose intolerance and in the insulin

Table 1 Clinical characteristics of subjects studied

Group	No.	M/F	Age (yr)	Height (cm)	Weight (kg)	Transferrin (μmol/l)	Fe (μmol/l)	Transferrin saturation (%)
Controls	6	4/2	51 ± 3	167 ± 4	73 ± 4	30.2 ± 0.9	14.2 ± 1.6	23 ± 2
Haemochromatosis								
Normal GTT	5	4/1	54 ± 5	171 ± 5	74 ± 8	24.4 ± 1.0	25.4 ± 2.1	59 ± 6
Abnormal GTT	7	6/1	47 ± 3	173 ± 3	64 ± 5	23.7 ± 1.1	22.0 ± 3.4	54 ± 5
Insulin-treated diabetes	4	4/0	54 ± 3	172 ± 3	72 ± 4	21.9 ± 1.5	27.8 ± 6.8	60 ± 9

Patients with idiopathic haemochromatosis were subdivided according to their respective glucose tolerance tests (GTT). Serum iron, transferrin were measured at the time of study and per cent saturation was calculated. Means ± SEM are given: M/F: males/females.

Fig. 1 Glucagon immunoreactivity (30-K) in plasma fractions of a patient with haemochromatosis and diabetes after Biogel P-30 column chromatography. Two millilitres of diluted (1:2) plasma (●—●) or of plasma treated with charcoal (v/v) (○---○) were applied to Biogel P-30 column chromatography. The fraction volume was 1 ml. Recovery: ●—● 117%, ○---○ 116%.

treated diabetics. Plasma glucagon, however, was significantly raised at 30 minutes after arginine administration in all patients with idiopathic haemochromatosis, regardless of whether or not they had carbohydrate intolerance (Table 2, Fig. 2A).

In some patients, however, glucagon immunoreactivity was not due exclusively to the 3500 MW fraction eluting with the glucagon-¹²⁵I marker. Figure 1 shows a representative elution profile of plasma from a patient with both haemochromatosis and insulin-requiring diabetes in whom only 19% of total plasma glucagon immunoreactivity was due to glucagon. The remaining glucagon immunoreactivity was associated with fractions of high

molecular weight. When the plasma was treated with charcoal adsorbing the smaller peptides such as glucagon, the big molecular weight moiety remained and the glucagon peak disappeared (Fig. 1).

Glucagon immunoreactivity was, therefore, measured in all plasma samples after charcoal treatment. Surprisingly, the remaining glucagon immunoreactivity associated with high molecular weight fractions was raised in all three groups of patients with idiopathic haemochromatosis (Fig. 2B): the most pronounced difference occurred in the patients with abnormal glucose tolerance. The third panel (C) of Fig. 2 depicts the calculated values for 'true' plasma glucagon obtained by subtraction of the values after charcoal treatment from those before charcoal exposure—that is, 'total' immunoreactive glucagon. This shows that the patients with idiopathic haemochromatosis exhibit similar plasma levels of 'true' glucagon, as do the normal control subjects (Fig. 2C).

Discussion

Although the results presented here confirm earlier findings (Gay *et al.*, 1968; Passa *et al.*, 1977) showing that in patients with idiopathic haemochromatosis and diabetes mellitus glucagon secretion is not diminished, they stress the importance of adequate methods in the measurement of plasma glucagon. Using these, it becomes evident that this form of diabetes is not associated with raised plasma levels of glucagon (Fig. 2C) as reported by the authors mentioned. This finding was unexpected as diabetic patients (Unger *et al.*, 1970; Muller *et al.*, 1970; Buchanan and McCarroll, 1972; Day and Anderson, 1973) as well as animals with insulin deficiency (Muller *et al.*, 1971; Frankel *et al.*, 1974) exhibit

Table 2 Effect of arginine (0.45 g/kg/min i.v.) on concentrations of glucose and immunoreactive glucagon (30-K; total) in plasmas of normal subjects (N = 6) and patients with idiopathic haemochromatosis (IH) with either normal glucose tolerance (GTT) (N = 5), pathological glucose tolerance (N = 7), or treated (Rx) with insulin (N = 4)

Minutes:	Arginine IV (minutes)									
	-20	-10	0	5	10	20	30	40	50	60
Glucose (mmol/l)										
Normal subjects	4.3 ± 0.3	4.3 ± 0.3	4.4 ± 0.3	5.0 ± 0.4	5.1 ± 0.3	5.5 ± 0.3	5.4 ± 0.4	5.2 ± 0.4	4.8 ± 0.3	4.3 ± 0.2
IH normal GTT	4.3 ± 0.5	4.4 ± 0.4	4.4 ± 0.4	4.6 ± 0.6	4.5 ± 0.6	4.8 ± 0.6	4.9 ± 0.3	5.4 ± 0.5	4.9 ± 0.9	4.7 ± 0.7
abnormal GTT	4.9 ± 0.2	5.3 ± 0.4	5.2 ± 0.4	5.3 ± 0.4	5.6 ± 0.5	6.0 ± 0.4	5.7 ± 0.4	5.5 ± 0.6	5.6 ± 0.6	5.1 ± 0.6
insulin Rx	9.2* ± 1.7	9.5* ± 2.2	9.4* ± 1.8	9.8* ± 2.1	10.1* ± 2.1	10.7* ± 2.1	11.1* ± 2.1	11.6* ± 2.1	11.6* ± 1.9	11.9* ± 2.3
Insulin (pmol/l)										
Normal subjects	93 ± 14	79 ± 14	86 ± 14	330 ± 29	266 ± 50	416 ± 108	502 ± 144	560 ± 158	352 ± 79	215 ± 36
IH normal GTT	93 ± 29	93 ± 29	72 ± 43	323 ± 151	194 ± 93	273 ± 122	316 ± 108	316 ± 100	316 ± 194	194 ± 93
abnormal GTT	100 ± 7	108 ± 7	108 ± 14	251 ± 36	187 ± 22	208* ± 14	230* ± 43	280* ± 29	237 ± 36	172 ± 22
Glucagon 30-K (pmol/l)										
Normal subjects	43 ± 10	38 ± 8	36 ± 7	67 ± 11	82 ± 13	26 ± 13	98 ± 10	107 ± 14	104 ± 10	94 ± 14
IH normal GTT	57 ± 11	58 ± 9	53 ± 6	92 ± 22	97 ± 11	147 ± 27	149* ± 18	153* ± 9	131 ± 17	116 ± 14
abnormal GTT	74* ± 12	74* ± 11	76* ± 9	113* ± 17	125* ± 21	147* ± 20	168* ± 29	166 ± 29	151 ± 25	119 ± 17
insulin Rx	61 ± 26	56 ± 17	50 ± 16	70 ± 15	101 ± 6	123 ± 15	135* ± 16	130 ± 15	123 ± 20	99 ± 23

Means ± SEM are given. The asterisks indicate significant differences ($P < 0.05$) when compared with the normal control group.

Fig. 2 Effects of intravenous arginine (450 mg/kg/40 min) on immunoreactive glucagon (IRG, antiserum 30-K) in plasma of patients with idiopathic haemochromatosis associated with normal glucose tolerance (▲), abnormal glucose tolerance (●), or insulin-requiring diabetes (○). The shaded area represents a normal control group (mean $\pm$ SEM). A: total IRG, B: IRG left after charcoal treatment of the plasma, C: the calculated values for true glucagon ($A - B = C$). Asterisks indicate significant differences ($P < 0.05$) between patient groups and the normal control group.

raised plasma concentrations of glucagon. Accordingly, those of our patients treated with insulin would have been expected to have raised glucagon levels. The fact that this was not the case suggests the possibility of a partial alpha-cell destruction by haemochromatosis. A possible argument against this is the fact that the alpha-cell destruction was only just sufficient to reduce plasma glucagon to levels indistinguishable from a normal control group. Histological examination of islets of patients with idiopathic haemochromatosis were, in fact, reported to have iron deposits exclusively in the beta-cells (Hartoft, 1956). On the other hand, the pancreatectomised patients demonstrate that diabetes mellitus does not require (Unger and Orci, 1975) the presence of glucagon (Barnes and Bloom, 1976; Muller *et al.*, 1979). Therefore, hyperglucagonaemia called 'characteristic' for diabetes mellitus (Unger, 1976) might not necessarily be present in all forms of diabetes.

The data show that patients with idiopathic haemochromatosis exhibit raised plasma levels of high molecular weight glucagon immunoreactivity (Figs 1 and 2). This factor of molecular weight greater than 30 000 (Weir *et al.*, 1975), cross-reacting with glucagon antiserum 30-K has been called 'big plasma glucagon' or 'BPG' (Valverde *et al.*, 1974).

It is as yet not well characterised: the fact that trypsin treatment can convert part of it into immunoreactive material close to the size of glucagon (Valverde *et al.*, 1974; Jaspan and Rubenstein, 1977) suggests that part of BPG might represent glucagon bound to a carrier. It might, on the other hand, represent a pre-pro-hormone or cross-reacting material independent on glucagon or a combination of these. Srikant *et al.* (1976) found that tissue-extracted BPG could induce glucose release from rat livers perfused *in vitro*. One might speculate that the increased γ -globulin in the serum of some of our patients was related to the increased plasma levels of BPG. In a patient with agammaglobulinaemia, absence of 'large molecular weight glucagon' has been reported (Von Schenk, 1977). An attempt, however, to correlate plasma BPG in our patients with the respective plasma transferrin concentrations was negative. Similarly, it is doubtful whether the presence or absence of diabetes, or its severity, in the patients with idiopathic haemochromatosis had any influence on the amount of BPG, as between these groups no significant difference was detected (Fig. 2B).

These results confirm that measurements of plasma glucagon using antiserum 30-K or similar antisera can be misleading (Weir *et al.*, 1975;

Valverde *et al.*, 1974; Jaspan and Rubenstein, 1977). States of hyperglucagonaemia diagnosed with such an antiserum have, therefore, to be qualified by methods which serve to eliminate cross-reacting high-molecular-weight substances before (Heding, 1971) or after the glucagon immunoassay.

In conclusion, patients with idiopathic haemochromatosis, whether or not associated with diabetes, have plasma levels of glucagon within the range of normal controls. These patients exhibit raised plasma concentrations of a big-molecular-weight glucagon immunoreactivity, an interesting finding, the significance of which remains to be investigated.

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