Hepatocyte Insulin Binding and Action in Rats with Sommatomammotrophic Tumours

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Summary. The effects of chronic elevations of growth hormone levels on hepatic insulin binding and action were studied in rats with subcutaneously implanted growth hormone-producing tumours. These animals were significantly heavier ($p < 0.001$; 388 ± 29 versus 239 ± 4 g), had elevated insulin levels, (6.8 ± 0.6 versus 3.3 ± 0.5 ng/ml), lower glucose concentrations (6.0 ± 0.4 versus 9.3 ± 0.5 mmol/l) and larger hepatocyte diameters (28.7 ± 0.6 versus 24.0 ± 0.4 μ m) and surface areas (2661 ± 119 versus 1835 ± 65 μ m²) than control rats. Insulin binding and action [net (C^{14})-glucose incorporation into glycogen] were compared in hepatocytes isolated from these two groups. Because of the difference in hepatocyte size, insulin binding was normalized for cell surface area. Binding of the tracer alone (0.9 ± 0.05 versus $1.3 \pm 0.12\%$ /cm²) and capacity of the high affinity, low capacity receptor (30.9 ± 5.9 versus

65.2 ± 5.9 sites/ μ m²) were significantly decreased ($p < 0.02$) in tumour-bearing rats. Dose-response curves of insulin action in hepatocytes chronically exposed to excess growth hormone were shifted to the right. The maximal response was also significantly decreased. However, the relation between the amount of insulin bound and the proportion of the maximum insulin effect obtained were similar in cells from the two groups. Thus, in rat hepatocytes chronically exposed to excess growth hormone, both a receptor and a post-receptor defect occur while the insulin receptor itself functions normally.

Key words: Growth hormone, prolactin, insulin binding, rat hepatocytes, GH₃ pituitary tumours, post-receptor defect, glycogen, receptor defect.

Growth hormone (GH) is a potent insulin antagonist; this has been demonstrated by its effects on carbohydrate metabolism when secreted in excess by pituitary tumours in acromegaly [1], when injected into normal cats [2], dogs [3, 4] or man [5–8] and when secreted physiologically 3–5 h post-prandially [9] or shortly after falling asleep [10]. Although the liver plays a central role in carbohydrate homeostasis, little data are available concerning the interaction between GH and insulin on hepatic carbohydrate metabolism. This study describes the effects of chronic GH secretion by a transplantable pituitary somatomammotropic tumour originating from GH₃ cells [11] on insulin binding and insulin action in isolated rat hepatocytes.

Material and Methods

GH₃ cells were supplied by the American Type Culture Collection (Bethesda, Maryland). These cells are an established clonal strain of rat pituitary tumour cells secreting both GH and prolactin [12]. Cells were maintained in monolayer culture in a humidified atmosphere of 95% air – 5% CO₂ using Dulbecco's modified Eagle's medium (DMEM) supplemented with horse serum (15%), fetal calf serum (2.5%), penicillin (50 U/ml), streptomycin (50 μ g/ml) and glutamine

(5 mmol/l) (Irvine Scientific, Santa Ana, California). For tumour generation, approximately $5\text{--}7 \times 10^6$ cells derived from the same parent culture were harvested by trypsinization, washed and resuspended in DMEM containing penicillin (50 U/ml) and streptomycin (50 μ g/ml). An aliquot of the cell suspension was counted in an automatic cell counter (Coulter Electronics, Hialeah, Florida), and the suspension was diluted to $\sim 10^6$ cells/ml. One ml was injected SC into female Wistar-Furth rats as described previously [11]. Tumours were palpable by the end of 4 weeks. Control rats were treated similarly except that they received the media alone. Tumour-bearing and control rats were sacrificed alternately 6–8 weeks after inoculation.

Rats were anaesthetized with sodium pentobarbital (60 mg/kg). A cannula was placed in the portal vein to prepare isolated hepatocytes (see below) but before the perfusion was started, blood was sampled from the inferior vena cava via a small needle attached to a syringe. Glucose concentrations were measured by a glucose oxidase method (Beckman glucose analyzer) and insulin [13], GH [11] and prolactin [11] by the appropriate radioimmunoassays. Materials for the rat GH and prolactin assays were kindly supplied by the National Institute of Arthritis, Diabetes and Kidney Disease, Pituitary Hormone Distribution Program, Bethesda, Maryland.

Hepatocyte Isolation

Fresh hepatocytes were isolated from the livers of the fed control and tumour rats by the method of Edwards [14], which utilizes a collagenase perfusion in situ based on the protocol of Berry and Friend [15].

After an initial perfusion with calcium-free Hank's solution, Swim's S-77 media (an amino acid-rich solution obtained from Grand Island Biological, Grand Island, New York) supplemented with NaHCO_3 (25 mmol/l), tricine buffer (50 mmol/l, pH 7.4), 2.5% bovine serum albumin, L-glutamine (279 mg/l) and L-cystine (12 mg/l) is the basic media used for both isolation of the cells and the insulin binding and action assays. The protocol described by Edwards [14] was modified by perfusing for 40 min and changing the collagenase concentration to 0.4 mg/ml. Cells were counted in a haemocytometer after staining with Trypan blue. Viability was usually >90% by Trypan blue exclusion and declined <10% during the course of any incubation. The diameter (d) of the hepatocytes was measured with a calibrated eyepiece and the surface area (SA) calculated using the formula for a sphere, $\text{SA} = \pi d^2$. Although hepatocytes have villi and are therefore not perfect spheres, the relative differences between the surface area of cells from tumour-bearing and control animals should be valid.

Insulin Action

Isolated hepatocytes ($0.5\text{--}1.0 \times 10^7$ cells/4 ml) were incubated in Swim's media with glucose (11.1 mmol/l), bacitracin (2.4 mmol/l), (^{14}C)-glucose (0.1 $\mu\text{Ci/ml}$), and beef insulin in the following amounts: 0, 1, 2, 4, 6, 8, 10, 20, and 40 ng/ml. Bacitracin was added to inhibit insulin degradation [16, 17] since liver is an active site for this and small amounts of insulin were used. All incubations were carried out in 25 ml polycarbonate Erlenmeyer flasks (Nalge, Rochester, New York) (initially gassed for 1–2 min with 95% O_2 /5% CO_2) at 37 °C for 1 h in a metabolic shaker (Lab-line Instruments, Melrose Park, Illinois) at approximately 100 oscillations/min. At the end of incubation, the medium was decanted into centrifuge tubes and spun at $50 \times g$ at 4 °C for 5 min. The supernatants were discarded and the cell glycogen extracted by the method of Van Handel [18]. The extracted glycogen was hydrolyzed [19] and the liberated glucose measured by the ferricyanide method [20] for determination of total cell glycogen. Because of variable counts in the intact glycogen molecule [19], an aliquot of the hydrolyzed extract was also counted for ^{14}C .

Iodination of Insulin

^{125}I -labelled insulin was prepared by enzymatic iodination as described by Morrison and Bayse [21] using Enzymobead reagent obtained from Bio-Rad Laboratories (Richmond, California). More than 95% of the radioactivity was precipitable by trichloroacetic acid and was recovered in the intact insulin peak when ^{125}I -insulin was subjected to gel filtration over G-50 Sephadex. The specific activity of the labelled preparations varied from 80–100 $\mu\text{Ci}/\mu\text{g}$ as determined by the displacement analysis procedure described by Verhoff et al. [22].

Binding Studies

Fresh hepatocytes ($0.8\text{--}1.2 \times 10^6$ cells/ml) were suspended in Swim's media, modified as above at pH 7.4, with (^{125}I)-insulin and unlabelled insulin in 12×75 mm polystyrene tubes with a final reaction volume of 0.5 ml. Insulin concentrations were 0, 1, 2, 4, 6, 8, 10, 20, 40, 100, 300, and 10^5 ng/ml. Incubations were carried out for 90 min at 20 °C with periodic shaking. Bacitracin (2.4 mmol/l), included in all incubations, has a minimal effect on insulin binding at 20 °C [23].

Incubations were terminated by removing aliquots from the reaction mixture and centrifuging in plastic microfuge tubes. Binding in the presence of 10^5 ng/ml of unlabelled insulin (non-specific binding) was limited to 5% of the total binding and was subtracted from the total binding to yield specific binding.

Data Analysis

Net (^{14}C)-glucose incorporation into glycogen was calculated from the initial specific activity of media glucose. There was no accumula-

tion of glycogen in these cells during incubation in agreement with other reports studying hepatocytes incubated with glucose [23–25].

The curvilinear Scatchard plot describing insulin binding was analyzed as representing two classes of receptors. Analysis of the two receptor model utilized the equation:

$$\frac{B}{F} = \frac{1}{2} \frac{[K_1(C_1 - B) + K_2(C_2 - B)] + \sqrt{[K_1(C_1 - B) - K_2(C_2 - B)]^2 + 4 K_1 K_2 C_1 C_2}}{2}$$

which was derived by Feldman [26] for a two-binding site, one-ligand model. In this equation, B is the bound hormone, F is the free hormone, K_1 and K_2 are the high and low affinity binding constants, and C represents the number of sites for the high affinity, low capacity (C_1) and low affinity, high capacity (C_2) receptors. The analyses were performed by an IBM 360 computer using the Biomedical program BMDP3R for non-linear least squares analysis. Binding affinities were evaluated also by comparing the amount of unlabelled insulin which competitively inhibited 50% of the tracer binding (ED_{50}).

The results in the tumour-bearing and control rats were expressed as mean $\pm$ SEM and compared by Student's t-test for unpaired means. Significance was accepted at the 0.05 level.

Results

The tumour-bearing rats were heavier, had larger hepatocytes, lower random glucose levels and higher insulin concentrations than the control group (Table 1). GH and prolactin levels were markedly increased in the experimental animals as expected.

Insulin Binding

Since insulin receptors are located on the cell surface, differences in hepatocyte size will influence the binding results. Therefore, insulin binding was normalized to cell surface area. Competitive binding curves are shown in Figure 1. Hepatocytes from tumour-bearing animals bound less insulin per unit of surface area at the lower insulin concentrations. Thus, it was not surprising that when the data were analyzed by Scatchard plots (Fig. 2) and binding parameters calculated (Table 2), liver cells from the tumour-bearing group showed a significant reduction of tracer binding and of the capacity of the high affinity, low capacity receptor. The similarity of affinities for both classes of receptors as calculated from the Scatchard plot was supported by the nearly identical ED_{50} values in the two groups.

There was a significant inverse correlation ($p < 0.025$, $r = -0.56$) between the insulin concentrations and the capacity of the high affinity, low capacity receptor in these 16 animals.

These alterations were due to changes in insulin binding. There was no difference in insulin degradation as assessed by the reduction in trichloroacetic acid precipitability during the binding assay in cells from control ($2.1 \pm 0.3\%$) and tumour-bearing ($1.7 \pm 0.2\%$) animals.

Insulin Action

Net (^{14}C)-glucose incorporation into glycogen in the absence of insulin was reduced, but not significantly dif-

Table 1. Characteristics of eight control and eight tumour-bearing rats

	Body weight (g)	Serum GH (ng/ml)	Serum prolactin (ng/ml)	Serum glucose (mmol/l)	Serum insulin (ng/ml)	Hepatocyte diameter (μm)	Hepatocyte surface area (μm^2)
Control rats	239 $\pm$ 4	36 $\pm$ 24 (n = 7)	55 $\pm$ 29 (n = 6)	9.3 $\pm$ 0.5	3.3 $\pm$ 0.5	24.0 $\pm$ 0.4	1835 $\pm$ 65
Tumour-bearing rats	388 $\pm$ 29	5065 $\pm$ 1325	4141 $\pm$ 694	6.0 $\pm$ 0.4	6.8 $\pm$ 0.6	28.7 $\pm$ 0.6	2661 $\pm$ 119
<i>p</i>	< 0.001	< 0.005	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Results expressed as mean $\pm$ SEM**Table 2.** Insulin binding parameters in hepatocytes isolated from eight control and eight tumour-bearing rats

	Tracer binding (%/cm ²)	K ₁ ^a (1/10 ⁻⁹ mol)	K ₂ (1/10 ⁻⁹ mol)	C ₁ (sites/ μm^2)	C ₂ (sites/ μm^2)	ED ₅₀ (ng/ml)
Control rats	1.26 $\pm$ 0.12	1.3 $\pm$ 0.1	0.05 $\pm$ 0.01	65.2 $\pm$ 5.9	297 $\pm$ 48	8.9 $\pm$ 0.6
Tumour-bearing rats	0.89 $\pm$ 0.05	1.0 $\pm$ 0.1	0.05 $\pm$ 0.01	30.9 $\pm$ 5.9	242 $\pm$ 67	9.0 $\pm$ 0.7
<i>p</i>	< 0.02	NS	NS	< 0.005	NS	NS

^a K₁ – affinity of the high affinity, low capacity receptor; K₂ – affinity of the low affinity, high capacity receptor; C₁ – capacity of the high affinity, low capacity receptor; C₂ – capacity of the low affinity, high capacity receptor; ED₅₀ – concentration of insulin displacing 50% of the tracer binding. NS = not significant

Fig. 1. Competitive insulin-binding curves in hepatocytes isolated from eight control (●—●) and eight tumour-bearing (○----○) rats **Fig. 2.** Scatchard plots of the data depicted in Figure 1. The variances are SEM

ferent, in hepatocytes from five tumour-bearing rats (9.3 ± 0.8 nmol/ 10^6 cells) compared with five control animals (17.8 ± 5.3 nmol/ 10^6 cells). The response to insulin, expressed as a percentage of this basal value, is shown in Figure 3. The decrease in cells from the tumour-bearing group was statistically significant at every insulin concentration except the highest. However, the maximal response for each animal was significantly lower ($p < 0.05$) for the five tumour-bearing ($156 \pm 8\%$) compared with the five control rats ($206 \pm 19\%$). Thus, responsiveness [27] of net insulin-stimulated (^{14}C)-glucose incorporation into glycogen in hepatocytes exposed to high levels of GH was significantly reduced.

The sensitivity [27] of this pathway to insulin was also examined by setting the maximal response in each animal to 100% and expressing the effects of smaller concentrations of insulin as a percentage of that maximal response. Figure 4 depicts this relationship and demonstrates a rightward shift of the dose-response curve in cells from the tumour-bearing rats. When the concentration of insulin which gives 50% of the maximal response was calculated for each animal, there was a significant increase in the tumour-bearing groups (6.8 ± 1.3 ng/ml) compared with the controls (2.6 ± 0.4 ng/ml; $p < 0.02$). Thus, the sensitivity of net insulin-stimulated (^{14}C)-glucose incorporation into glycogen in

Fig. 3. Dose-response curves of the insulin effects on net (^{14}C)-glucose incorporation into glycogen in hepatocytes isolated from five control (●—●) and five tumour-bearing (○----○) rats expressed as a percentage of the basal (no added insulin) value. The SE of each value is 9% or less of the mean. (*** $p < 0.001$, ** $p < 0.005$, * $p < 0.02$, * $p < 0.05$)

Fig. 4. Relationship between the extracellular insulin concentration and the percentage of the maximal insulin effect in hepatocytes isolated from five control (●—●) and five tumour-bearing (○----○) rats

Fig. 5. Relationship between the amount of bound insulin and the percentage of the maximal insulin effect in hepatocytes isolated from five control (●—●) and five tumour-bearing (○----○) rats

hepatocytes exposed to high levels of GH was also significantly decreased [27].

To determine if the insulin receptors in the hepatocytes from the tumour-bearing animals are functionally normal, the amount of insulin bound was plotted against the percentage of the maximum insulin effect at

tained (Fig. 5). The similarity of this relationship in liver cells from control and experimental animals suggests that the insulin receptor on hepatocytes chronically exposed to excess GH functions normally.

Discussion

The characteristics of these somatomammotrophic pituitary tumour-bearing rats are similar to previous reports describing animals harbouring GH-producing neoplasms. Rats exposed to excess GH weigh more, whether fed ad libitum [28, 29] or pair fed with control animals [30]. Increased hepatocyte size in our rats is consistent with the increase in liver weight [28, 29]. This increase in organ weight is not accounted for by an increased water content [28]. Furthermore, in rats carrying GH-secreting tumours, lower glucose concentrations, especially after a glucose challenge [30, 31], are associated with high circulating insulin levels [28, 30, 31], increased pancreatic islet mass [30, 31], increased pancreatic insulin content [28, 30] and increased insulin secretion by pancreatic slices and isolated islets [30]. Thus, although excess GH causes insulin antagonism in the intact rat, the ability of the pancreas to secrete additional insulin prevents the development of hyperglycaemia.

It is important to assess the possible role of the high prolactin levels before ascribing the observed insulin antagonism to GH in these tumour-bearing rats. Although some patients with elevated prolactin concentrations have mild glucose intolerance associated with increased insulin levels [32, 33], neither intravenous glucose challenges in hyperprolactinaemic patients [34] nor studies in dogs [35] or rats [36] suggest that prolactin affects insulin action. Therefore, in view of the well documented profound insulin antagonism induced by GH [1–10], it seems reasonable to conclude that the changes observed in these tumour-bearing rats are predominantly exerted by GH.

The available information concerning the effect of GH on insulin binding is somewhat controversial. In studies utilizing liver membranes prepared from normal or hypophysectomized rats injected with GH [37] or monocytes isolated from acromegalic patients [38], a decreased binding capacity was offset by an increased binding affinity so that little change in total insulin binding was noted. In other studies in which GH was administered to children with hypopituitarism [39] or to normal subjects [8, 40], no changes in insulin-binding capacities or affinities were observed.

Chronic GH excess in our tumour-bearing rats was associated with a significant decrease in both tracer insulin binding and the capacity of the high affinity, low capacity receptor. Since GH itself does not directly affect insulin binding [38, 41, 42] and hyperinsulinaemia characterizes states of GH excess, e.g. in acromegaly [1, 38], GH-injected dogs [4] and humans [6, 8] and GH-producing tumours in rats [28, 30, 31], the diminished

insulin binding in these cells probably reflects down regulation of the insulin receptor [43]. This suggestion is supported by the significant inverse correlation between insulin concentrations and the capacity of the high affinity, low capacity receptor in these 16 rats.

Only a few studies have examined the chronic effect of GH (i.e., at least 4 h of exposure to the hormone) on hepatic carbohydrate metabolism. The ability of insulin [4, 44, 45] or glucose [46] to suppress glucose production was impaired and insulin-stimulated incorporation of radioactive glucose into glycogen and non-glycogen constituents was virtually eliminated by GH [44]. Insulin-stimulated glycogen synthesis in diaphragms isolated from rats with GH-producing tumours was also markedly suppressed [28].

Our in-vitro results with hepatocytes isolated from the tumour-bearing rats are consistent with these published studies. The insulin dose-response curve on net (^{14}C)-glucose incorporation into glycogen was not only shifted to the right but the maximal response over the basal level was depressed. The former denotes impaired sensitivity and the latter decreased responsiveness to insulin [27]. Since hepatocytes have spare insulin receptors [23], the diminished capacity of the high affinity, low capacity receptor can explain the impaired sensitivity. The decreased responsiveness is due to a post-receptor defect [27].

Insulin binding was normalized to cell surface area in these experiments as suggested for adipocytes [47–49] and hepatocytes [50] in studies where experimental and control cells differed in size. Furthermore, the greatest constancy of insulin binding capacity among various cell systems is achieved when the data are expressed on the basis of surface area [51]. However, if the data in these studies were calculated on a per cell basis, there are no differences in binding between control and tumour-bearing animals. If this approach were used, the impaired sensitivity would reflect the post-receptor defect. Such a situation has been described [52].

The relation between the amount of insulin bound and the biological effect attained signifies whether the signal generated by the hormone-receptor complex is transmitted normally. In the present study, comparable effects of insulin occurred in liver cells from control and tumour-bearing animals for a given amount of insulin bound. Therefore, the effect of chronic GH excess on hepatocyte insulin receptors is quantitative rather than qualitative. That is, although the number of insulin receptors is decreased, the ones present function appropriately.

Hepatocytes from obese rats also show receptor and post-receptor defects [53]. However, the results in this study are due to excess GH, not obesity, since animals harbouring GH-producing tumours have very little fat compared with control rats [29]. The increased weight of these animals is lean body mass [29].

In conclusion, rat hepatocytes *chronically* exposed to excess GH demonstrate: (a) decreased capacity of

the high affinity, low capacity receptor, probably secondary to down regulation; (b) decreased insulin sensitivity, probably secondary to the diminished binding; (c) impaired responsiveness to insulin; and (d) normal functioning of the insulin receptor. Therefore, both a receptor and post-receptor defect occur. However, since subjects exposed to excess GH for short, intermittent periods of time demonstrate insulin antagonism in the face of normal insulin binding [8, 39, 40], we suggest that the post-receptor defect causing decreased tissue responsiveness occurs earlier and is more important in altering carbohydrate metabolism than the receptor defect causing impaired tissue sensitivity.

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