Glucagon resistance of hepatoma cells

Evidence for receptor and post-receptor defects

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Of all available liver cells in culture, only primary cultured hepatocytes are known to respond to glucagon *in vitro*. In the present study we investigated whether glucagon could stimulate amino acid transport and tyrosine aminotransferase (TAT; EC 2.6.1.5) activity (two well-characterized glucagon effects in the liver) in Fao cells, a highly differentiated rat hepatoma cell line. We found that glucagon had no effect on transport of α -aminoisobutyric acid (AIB; a non-metabolizable alanine analogue) nor on TAT activity, even though both activities could be fully induced by insulin [2-fold and 3-fold effects for AIB transport and TAT activity, respectively, after 6 h; EC₅₀ (median effective concentration)=0.3 nM], or by dexamethasone (5–8-fold effects after 20 h; EC₅₀ = 2 nM). Analysis of [¹²⁵I]iodoglucagon binding revealed that Fao cells bind less than 1% as much glucagon as do hepatocytes, whereas insulin binding in Fao cells was 50% higher than in hepatocytes. The addition of dibutyryl cyclic AMP, which fully mimics the glucagon stimulation of both AIB transport and TAT activity in hepatocytes, induced TAT activity in Fao cells (a 2-fold effect at 0.1 mM-dibutyryl cyclic AMP) but had no effect on AIB transport. Cholera toxin stimulated TAT activity to the same extent as did dibutyryl cyclic AMP. These results indicate that the lack of glucagon responsiveness in cultured hepatoma cells results from both a receptor defect and, for amino acid transport, an additional post-receptor defect. Moreover, the results show that amino acid transport and TAT activity, which appeared to be co-induced by insulin or by dexamethasone in these cells, respond differently to cyclic AMP. This suggests that different mechanisms are involved in the induction of these activities by glucagon in liver.

The liver is the major target organ for glucagon action. Glucagon has been shown to bind to specific receptors in purified liver plasma membranes (Rodbell *et al.*, 1971) and in freshly isolated hepatocytes in suspension (Sonne *et al.*, 1978; Fehlmann *et al.*, 1981) or in primary monolayer culture (Morin *et al.*, 1982). Addition of glucagon to hepatocytes is rapidly followed by an increase in intracellular cyclic AMP (Rosselin *et al.*, 1974; Sonne *et al.*, 1978), an increased permeability to K⁺ (Fehlmann & Freychet, 1981) and membrane hyperpolarization, an increased glucose production (Exton & Park, 1968;

Friedmann & Dambach, 1980), and, after a lag period, an increase in amino acid transport (Pariza *et al.*, 1976; Fehlmann *et al.*, 1979) and TAT activity (Ernest *et al.*, 1977; Gurr & Potter, 1980). In addition to these metabolic effects, glucagon is also thought to play an important 'hepatotrophic' role. Experiments *in vivo* and *in vitro* have shown that glucagon is essential for the induction of thymidine incorporation into hepatic DNA (Richman *et al.*, 1976; Koch & Leffert, 1979) and for liver regeneration after partial hepatectomy (Bucher & Swaffield, 1975; Leffert *et al.*, 1975). A better understanding of the respective actions of glucagon on liver metabolism and growth, and the possible interactions between these actions, would require studies *in vitro* in hepatocytes maintained in tissue culture. Unfortunately, primary hepatocytes do not grow well, cannot be maintained for long period in

Abbreviations used: TAT, tyrosine aminotransferase (EC 2.6.1.5); AIB, α -aminoisobutyric acid.

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culture and are often contaminated by non-liver parenchymal cells (Koch & Leffert, 1980). Alternative approaches to the study of liver cells are the use of hepatocytes after spontaneous transformation (Beckner *et al.*, 1980) or the use of different hepatoma cell lines (Kelley *et al.*, 1978; Mirel *et al.*, 1978; Pezzino *et al.*, 1979). Hepatoma cells have indeed been shown to grow rapidly in tissue culture and to maintain many differentiated hepatic functions such as albumin secretion, specific isoenzymes of alcohol dehydrogenase and aldolase, and basal and hormone-inducible TAT activity (Deschatrette & Weiss, 1974). Surprisingly, most of these liver cells do not respond to glucagon for TAT induction (C. R. Kahn, unpublished work) or for amino acid-transport stimulation (Kelley *et al.*, 1978). In the present work, we have studied in detail glucagon binding and action in Fao cells, a well-differentiated hepatoma cell line, and compared them with those in normal hepatocytes. We find that the lack of glucagon responsiveness in the hepatoma cell is due to receptor and post-receptor defects.

Materials and methods

Chemicals

α -Amino[1- ^{14} C]isobutyric acid ([^{14} C]AIB; sp. radioactivity 60 Ci/mol), [^{3} H]inulin (sp. radioactivity 2.2 Ci/mol), [^{125} I]iodoglucagon (sp. radioactivity 160 μ Ci/ μ g) and Na ^{125} I were purchased from New England Nuclear (Boston, MA, U.S.A.). Pig monocomponent insulin and pig glucagon were gifts from E. Lilly (Indianapolis, IN, U.S.A.). Coon's modified Ham's F12 culture medium and foetal-calf serum were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.). All other reagents were of the best grade commercially available.

Cells and incubation procedures

Fao is a well-differentiated clonal line of rat hepatoma cells derived from the H4-II-EC₃ line (Pitot *et al.*, 1964; Deschatrette & Weiss, 1974) established from a Reuber (1961) H-35 minimal-deviation hepatoma. This cell line was kindly provided by Dr. M. Weiss (Gif-sur-Yvette, France) and exhibits a number of liver-specific properties, including an inducible TAT activity, the secretion of rat serum albumin and the synthesis of liver-specific isomers of alcohol dehydrogenase and aldolase (Deschatrette & Weiss, 1974). In addition, Fao cells are resistant to both 8-azaguanine and ouabain (Deschatrette & Weiss, 1974). The cells were cultivated in a modified Ham's F12 medium (Coon & Weiss, 1969) supplemented with 5% (v/v) foetal-calf serum. For all experiments cells grown attached to the surface of Falcon plastic bottles were detached with a solution of trypsin (0.5 mg/ml) in 0.6 mM-EDTA/137 mM-NaCl/5 mM-KCl/5 mM-glu-

cose/7 mM-NaHCO₃ and replated in six-well (35 mm diameter) Corning plastic dishes. At confluency the culture medium was replaced by 0.9 ml of serum-free medium containing 1% bovine serum albumin. Cells were incubated for 16–24 h before addition of hormones. Hormones or cyclic AMP were added in 0.1 ml of the same medium and incubations were continued for various periods of time at 37°C.

Amino acid transport and TAT activity assays

To compare the hormonal regulation of amino acid transport and TAT activity under identical conditions, we have measured concomitantly AIB influx in cells of the same 35 mm well. The assay was initiated by aspirating the culture medium and by adding to cells 1 ml of amino acid-free Krebs–Ringer bicarbonate buffer (120 mM-NaCl/4.8 mM-KCl/1.2 mM-MgSO₄/24 mM-NaHCO₃/1.3 mM-CaCl₂/1.2 mM-K₂HPO₄, pH 7.4) containing [^{14} C]AIB (0.2 μ Ci/well), unlabelled AIB (final concn. 0.1 mM) and [^{3} H]insulin (1 μ Ci/well). After 15 min at 37°C, the reaction was terminated by aspirating the medium and by washing the plates twice with 4 ml of chilled saline (0.9% NaCl). Cells were detached in 1 ml of chilled saline by using a rubber policeman and sonicated for 10 s. A 500 μ l sample of the homogenate was counted for ^{3} H and ^{14} C radioactivity. AIB-transport data were corrected for extracellular trapping, by using [^{3} H]insulin as an extracellular marker. TAT activity was assayed directly with 20 μ l of the same homogenate by the Diamondstone (1966) method. Protein was determined by the method of Bradford (1976). TAT specific activity was expressed as units (1 unit of TAT catalyses the formation of 1 μ mol of *p*-hydroxyphenylpyruvate/min at 37°C) per mg of cell protein.

Insulin and glucagon binding

Binding assays were performed at 15°C in Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin and bacitracin (0.8 mg/ml). In hepatocytes, the binding assays were performed as previously described (Fehlmann *et al.*, 1981). In Fao cells binding assays were performed as follows. The culture medium was aspirated and replaced by 0.9 ml of Krebs–Ringer bicarbonate buffer. The cells were allowed to equilibrate at 15°C for 15 min and the assay was initiated by 0.1 ml of [^{125} I]iodoinsulin (20 pM) or 0.1 ml of [^{125} I]iodoglucagon (100 pM) and various concentrations of the corresponding unlabelled hormone. After 3 h incubation, the plates were washed twice with 4 ml of chilled 0.9% NaCl and the cells were solubilized in 0.01% sodium dodecyl sulphate and counted for radioactivity in a Tracer 1190 Autogamma counter. Non-specific binding was determined in the presence of 2 μ M unlabelled hormone.

Results

Effect of insulin on AIB transport and TAT activity in Fao cells

Incubation of Fao cells with 100 nM-insulin resulted in a progressive increase in AIB transport and TAT activity (Fig. 1a). Maximal stimulation of AIB uptake (measured under initial-velocity conditions in 15 min assays) was obtained after 6 h of cell exposure to insulin and represented a 2-fold increase above basal (Fig. 1). TAT activity increased linearly with time of cell exposure to insulin up to 5 h and then continued to increase at a slower rate. After 8 h, the increase in TAT activity represented a 4-fold increase above basal activity (Fig. 1a).

AIB transport and TAT activity stimulation were then studied after 6 h of cell exposure to various concentrations of insulin. The two dose-response curves were virtually superimposable (Fig. 1b). For

both activities, a maximal effect was observed with 10 nM-insulin and the half-maximal stimulation with approx. 0.5 nM-insulin.

Effect of dexamethasone on AIB transport and TAT activity in Fao cells

Incubation of Fao cells with 0.1 μ M-dexamethasone resulted in a progressive increase in AIB transport and TAT activity (Fig. 2a). Stimulation of both activities could be detected after 4 h of cell exposure to the synthetic glucocorticoid and then increased almost linearly with time. After 24 h of incubation, AIB influx was increased by 3–4-fold and TAT activity by approx. 7-fold (Fig. 2a). The concentration-response curves (Fig. 2b) showed that the maximal response was obtained with 100 nM-dexamethasone and the half-maximal res-

Fig. 1. Time course (a) and dose-responses (b) of insulin stimulation of AIB influx and TAT activity in Fao hepatoma cells

AIB influx and TAT activity were determined in the same cells, as indicated in the Materials and methods section. (a) Cells were incubated at 37°C for the indicated periods of time in the presence of 100 nM-insulin. Results represent the means $\pm$ S.E.M. for triplicate determinations. (b) Cells were incubated for 6 h at 37°C in the presence of the indicated concentrations of insulin. Results are expressed as percentages of the maximal stimulation of AIB influx or TAT activity.

Fig. 2. Time course (a) and dose-responses (b) of dexamethasone stimulation of AIB influx and TAT activity in Fao hepatoma cells

AIB influx and TAT activity were determined in the same cells (see the Materials and methods section). (a) Cells were incubated at 37°C for the indicated periods of time in the presence of 100 nM-dexamethasone. Results represent the means $\pm$ S.E.M. for triplicate determinations. (b) Cells were incubated for 24 h at 37°C in the presence of the indicated concentrations of dexamethasone. Results are expressed as percentages of the maximal stimulation of AIB influx or TAT activity.

ponse with 1–2 nM-dexamethasone for both activities. For dexamethasone concentrations higher than 1 μ M, the stimulation of AIB transport and TAT activity was less than the maximal stimulation, giving rise to biphasic dose-response curves.

Effect of glucagon on AIB transport and TAT activity in Fao cell

In contrast with insulin and dexamethasone, 100 nM glucagon had no effect on AIB influx (Table 1). Increasing the glucagon concentration to 1 μ M or the time of incubation with the hormone gave similar results (not shown). Glucagon was also ineffective in stimulating TAT activity (Table 2), whereas insulin induced the enzyme by 3.5-fold and dexamethasone by 8-fold.

Table 1. *Hormonal regulation of AIB influx in Fao hepatoma cells*

Cells were incubated for 24 h with dexamethasone or for 6 h with insulin, glucagon or dibutyryl cyclic AMP. AIB influx was measured in 15 min assays. Results from two individual experiments are presented.

Additions	AIB influx (nmol/min per mg of protein)	
None	0.15	0.13
Insulin (100 nM)	0.32	0.25
Dexamethasone (100 nM)	0.63	0.61
Glucagon (100 nM)	0.15	0.12
Dibutyryl cyclic AMP (1 mM)	0.14	0.11

Glucagon and insulin binding to Fao hepatoma cells and freshly isolated hepatocytes

As reported previously (Sonne *et al.*, 1978; Fehlmann *et al.*, 1981), glucagon binds to freshly isolated hepatocytes (Fig. 3a). At tracer concentrations (0.1 nM), about 5% of the glucagon was bound per mg of cell protein. Under identical conditions, on the other hand, Fao cells bind very little glucagon (Fig. 3). At tracer [^{125} I]iodoglucagon

Table 2. *Hormonal regulation of TAT activity in Fao hepatoma cells*

Cells were incubated for 24 h with dexamethasone or for 6 h with insulin, glucagon or dibutyryl cyclic AMP (Expt. I) or for 4 h with dibutyryl cyclic AMP or cholera toxin (Expt. II). TAT activity was determined as described in the Materials and methods section. Results from two individual determinations are presented.

Expt. I	Additions	TAT activity (unit/mg of protein)	
Expt. I	None	0.08	0.07
	Insulin (100 nM)	0.27	0.25
	Dexamethasone (100 nM)	0.60	0.62
	Glucagon (100 nM)	0.10	0.08
	Dibutyryl cyclic AMP (100 μ M)	0.20	0.18
Expt. II	None	0.09	0.08
	Dibutyryl cyclic AMP (50 μ M)	0.14	0.16
	Cholera toxin (0.3 μ g/ml)	0.14	0.16

Fig. 3. *Glucagon and insulin binding to isolated hepatocytes (●) and Fao hepatoma cells (○)*

Cells were incubated at 15°C for 2 h with 100 pM-[^{125} I]iodoglucagon or for 3 h with 30 pM-[^{125} I]iodoinsulin (steady-state conditions) with various concentrations of the corresponding unlabelled hormone. Non-specific binding was determined in the presence of 2 μ M-unlabelled hormone. Results are expressed as the percentage of [^{125} I]iodoinsulin specifically bound per mg of cell protein; 1 mg of hepatocyte protein corresponds to 0.67×10^6 cells and 1 mg of Fao-cell protein corresponds to 5×10^6 cells.

concentrations, Fao cells bind 200 times less glucagon than do hepatocytes when normalized for the same amount of cell protein. In contrast with glucagon binding, Fao cells bind 50% more insulin per mg of cell protein than do hepatocytes (Fig. 3b). Scatchard analysis of these data (results not shown) revealed that the higher insulin binding in Fao cells was due to a higher number of insulin receptors per mg of protein. When the results were expressed per 10^6 cells, hepatocytes actually possess 4–5 times more insulin receptors than do Fao cells, since they are much larger cells.

Effect of dibutyryl cyclic AMP on AIB transport and TAT activity in Fao cells

In Fao hepatoma cells, dibutyryl cyclic AMP, over the concentration range $1\mu\text{M}$ – 1mM , had no effect on AIB influx (Table 1). Similar results were obtained with 8-bromo cyclic AMP (results not shown). In marked contrast, dibutyryl cyclic AMP increased TAT activity in the same cells by approx. 2-fold (Table 2). Cholera toxin, which is known to stimulate adenylate cyclase in a variety of systems, produced a stimulation of TAT activity similar to that with dibutyryl cyclic AMP (Table 2).

Discussion

Studies of differentiated cell functions are often hampered by the fact that normal differentiated mammalian cells tend to de-differentiate in culture. However, many tumour-derived cell lines express differentiated functions characteristic of their tissue of origin and thus can be used as model systems for studying various regulatory aspects of cell metabolism and growth. In the present study, we have used Fao cells, a highly differentiated rat hepatoma cell line (Deschatrette & Weiss, 1974) to investigate the hormonal regulation of amino acid transport and TAT activity, two processes known to be under hormonal regulation in the normal liver (Pariza *et al.*, 1976; Ernest *et al.*, 1977; Fehlmann *et al.*, 1979; Gurr & Potter, 1980).

Induction of TAT activity by dexamethasone has been extensively investigated in rat liver and several rat hepatoma cell lines (Thompson *et al.*, 1966; Tomkins *et al.*, 1969; Deschatrette & Weiss, 1974; Olson *et al.*, 1980; Ruiz-Bravo & Ernest, 1982) and is often used to demonstrate the maintenance of hepatic functions in cultured liver cell systems (see Gurr & Potter, 1980). Glucocorticoids are thought to increase the synthesis of the enzyme by increasing the transcription of the specific gene(s) (Tomkins *et al.*, 1969; Olson *et al.*, 1980). On the other hand, the effect of glucocorticoids on amino acid transport has not been characterized in detail. Dexamethasone stimulates AIB transport in hepatocytes and H35 hepatoma cells (Kelley *et al.*, 1978;

Canivet *et al.*, 1980), but markedly inhibits AIB transport in HTC hepatoma cells (Kelley *et al.*, 1978; Heaton & Gelehrter, 1980; Heaton *et al.*, 1980). In Fao hepatoma cells, we found that dexamethasone stimulates TAT activity with a similar time- and dose-dependence to that for AIB-transport stimulation, which suggests a co-induction of the two activities. This is similar to that previously described to occur *in vivo* in response to glucagon or to cyclic AMP (Scott *et al.*, 1970). Moreover, these results demonstrate that Fao cells exhibit a high degree of hepatic differentiation, like H35 cells from which they have been derived, and clearly differ from HTC cells, a less differentiated hepatoma cell line.

Insulin action has been studied to some extent in both freshly isolated hepatocytes (Fehlmann *et al.*, 1979, 1981; Morin *et al.*, 1982) and hepatoma cells. Differentiated clones derived from the Reuber H35 rat hepatoma and HTC cells have been shown to display a high binding capacity for insulin (Pezzino *et al.*, 1979; Heaton *et al.*, 1980; Hofmann *et al.*, 1980; Heaton & Gelehrter, 1981; Iwamoto *et al.*, 1981). Moreover, insulin stimulation of amino acid transport (Kelley *et al.*, 1978; Iwamoto *et al.*, 1981) and TAT activity (Heaton *et al.*, 1980; Iwamoto *et al.*, 1981) has been demonstrated in both HTC and H4 cells and, in addition, differentiated clones derived from H4 cells have been shown to respond to insulin in terms of glycogen synthesis (Hofmann *et al.*, 1980). We have confirmed and extended these observations using Fao hepatoma cells derived from the H4 hepatoma. These cells display a high binding capacity for insulin and are highly sensitive to the hormone for both TAT activation and amino acid-transport stimulation. In addition, in these cells insulin also stimulates glycogen synthase, glucose incorporation into glycogen and Rb^+ uptake, induces a marked down-regulation of its receptors (M. Crettaz & C. R. Kahn, unpublished work), and stimulates insulin-receptor phosphorylation (Kasuga *et al.*, 1982). Thus this cell line provides a very useful model for further studies of the hepatic action of insulin.

In contrast with the multitude of responses of Fao cells to dexamethasone and insulin is their total unresponsiveness to glucagon. The lack of glucagon responsiveness in hepatoma cells was first reported by Mirel *et al.* (1978) for adenylate cyclase activation in two poorly differentiated hepatomas and by Kelley *et al.* (1978) for amino acid transport stimulation in four other hepatoma cell lines, including H4-II-EC₃, an ascendant of Fao cells (Deschatrette & Weiss, 1974). Our study revealed that glucagon-insensitivity of Fao cells is primarily due to an extremely low binding capacity of these cells for the hormone. These results therefore confirm previous observations that glucagon bind-

ing is decreased in plasma membranes of various hepatomas (Mirel *et al.*, 1978; Pezzino *et al.*, 1979).

The dramatic decrease in glucagon binding, however, does not appear to be the sole cause for the glucagon-insensitivity of Fao cells, since these cells also respond poorly to cyclic AMP in terms of TAT induction. Also, Byus & Fletcher (1982) have demonstrated an increase in cyclic AMP-dependent protein kinase activity in the parent Reuber hepatoma (H35) in response to glucagon. The mechanism of action of cyclic AMP on the induction of specific proteins in the liver is poorly understood. Cyclic AMP is thought to stimulate TAT activity at both a transcriptional and a post-transcriptional level (Snoek *et al.*, 1981; Noguchi *et al.*, 1982; Ruiz-Bravo & Ernest, 1982) and to stimulate amino acid transport by inducing the synthesis of high-affinity transport proteins (Fehlmann *et al.*, 1979). Given the relative insensitivity of Fao cells to cyclic AMP and the possibility of selectively isolating Fao variants characterized by different extents of cell differentiation (Moore & Weiss, 1982), the use of this cell system should help to shed some light on the mechanism of cyclic AMP action on protein expression in the liver.

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