Effects of growth factors on hormonal stimulation of amino acid transport in primary cultures of rat hepatocytes

Patrick AUBERGER, Michel SAMSON and Alphonse LE CAM

Institut National de la Santé et de la Recherche Médicale, Unité de Recherches sur les Hormones Polypeptidiques et la Physiopathologie Endocrinienne (I.N.S.E.R.M. U 145), Faculté de Médecine, Chemin de Vallombrose, 06034 Nice Cédex, France

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In primary cultures of rat hepatocytes, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and foetal-calf serum (FCS) prevented the stimulation of amino acid transport by glucagon (cyclic AMP-dependent) and by catecholamines (cyclic AMP-independent), but not by insulin. The insulin effect, as well as the effect of other hormones, were totally inhibited by thrombin through a mechanism independent of its proteolytic activity. The inhibitory effect of growth factors, not found in freshly isolated hepatocytes, was expressed very early in culture (4 h). Induction of tyrosine aminotransferase by glucagon or dexamethasone, which, like stimulation of transport, represents a late hormonal effect, was not affected by EGF, PDGF or FCS, but was inhibited by thrombin. In contrast, none of the rapid changes in protein phosphorylation caused by hormones was altered by growth factors. Thus the inhibition by growth factors of hormonal stimulation of transport presumably involves late step(s) in the cascade of events implicated in this hormonal effect.

Recent studies from this laboratory led to the intriguing finding that EGF inhibits the stimulation of amino acid transport by glucagon in primary cultures of adult rat hepatocytes (Morin *et al.*, 1981). This effect, which was not accounted for by inhibition of hormone binding by EGF (Fehlmann *et al.*, 1981), appeared rather paradoxical. Indeed, besides its well known growth-promoting properties in a wide variety of cell types (Carpenter & Cohen, 1979), EGF has been shown to stimulate DNA synthesis in cultured hepatocytes, an effect markedly potentiated by insulin and glucagon (Richman *et al.*, 1976; Bucher *et al.*, 1978; Koch & Leffert, 1979; Draghi *et al.*, 1980; McGowan *et al.*, 1981). We do not presently understand the complex mechanisms whereby hormones like insulin, glucagon and catecholamines stimulate amino acid transport in liver. It appears, however, that elucidation of the molecular basis of the inhibitory effect of EGF on the induction of amino acid transport by hormones should help to unravel these mechanisms. We have undertaken the present study to answer two questions. First, do other growth factors like PDGF, thrombin and those present in FCS share the

inhibitory property of EGF? Secondly, is the inhibitory effect restricted to the specific stimulation of amino acid transport by hormones?

Materials and methods

Isolation and culture of hepatocytes

Hepatocytes were isolated from adult male Wistar rats by collagenase dissociation of the liver as previously described (Le Cam *et al.*, 1976). Cells were plated on collagen-precoated plastic culture dishes and allowed to attach to the substratum for 4 h at 37°C in Waymouth's medium containing 10% (v/v) FCS (Morin *et al.*, 1982). The medium was then replaced by serum-free Waymouth's medium containing 0.2% (w/v) defatted BSA. Monolayers were then incubated for an additional 20 h in the presence of 100 units of penicillin/ml and 100 mg of streptomycin/ml.

Transport studies and enzyme induction experiments

For transport studies, cells were first washed three times in 1.5 ml of Krebs–Ringer bicarbonate (KRB) buffer. They were then incubated for 3 h at 37°C in KRB buffer containing 1% (w/v) defatted BSA, 0.8 mg of bacitracin/ml and 50 µg of gentamycin/ml, in the absence or presence of effectors (hormones, growth factors). Transport assays were then carried

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FCS, foetal-calf serum; BSA, bovine serum albumin; AIB, α -amino-[1-¹⁴C]isobutyrate.

out over 15 min as described previously (Morin *et al.*, 1981).

Induction of tyrosine aminotransferase (EC 2.6.1.5) by hormones was studied in 24 h-monolayers of hepatocytes. After exposure to the effectors for 6 h in KRB medium, cells were washed three times in 125 mM-phosphate buffer containing 1 mM-EDTA and 1 mM-dithiothreitol, detached by scraping with a rubber policeman, and resuspended in 0.5 ml of buffer. Hepatocytes were immediately sonicated and the extracts centrifuged at 20000 *g* for 30 min at 4°C. Enzyme activity was assayed in the supernatant as described by Granner & Tomkins (1970). Proteins were assayed by the method of Lowry *et al.* (1951). Enzyme activity was expressed as m-units/mg of cellular protein.

Phosphorylation experiments

Cells cultured for 24 h were extensively washed in phosphate-free KRB medium. They were then incubated for 1 h with [32 P]P_i (50 μ Ci/ml) in phosphate-free KRB medium supplemented with defatted BSA, bacitracin and antibiotics (Le Cam, 1982). The effectors (hormones, growth factors) were added and incubations were continued for 15 min. Incubations were stopped by aspirating the radioactive medium; cells were rapidly lysed in 0.5 ml of a boiling sodium dodecyl sulphate solution [3% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol and 10 mM-KH₂PO₄]. The samples were boiled for 5 min after addition of 2% (v/v) β -mercaptoethanol and 0.01% (w/v) Bromophenol Blue. Phosphoproteins present in total cell lysates were analysed by one-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described previously (Le Cam, 1982) using a 5–15% linear gradient of acrylamide as a separating gel. Gels were stained with Coomassie Brilliant Blue [0.25% (w/v) in 50% trichloroacetic acid] destained in 7% acetic acid and vacuum-dried. Autoradiograms were obtained after exposing the gels to Kodak X-Omat films.

Chemicals

AIB (sp. radioactivity 60 Ci/mol) and [32 P]P_i (carrier-free) (sp. radioactivity 5000 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Dexamethasone was from Roussel-Uclaf. Porcine insulin and glucagon (mono-components) were gifts from Novo [Novo Research Institute, Copenhagen, Denmark; and Novo (France), Paris, France]. EGF was from Collaborative Research and PDGF was a gift from Dr. J. Pouyssegur (Nice) (partially purified preparation from human platelets). Highly purified human thrombin (about 2600 units/mg) were generously supplied by Dr. John Fenton, II, Division of Laboratories and Research, New York State Depart-

ment of Health, Albany, NY, U.S.A., via Dr. Jacques Pouyssegur, Nice, France. Hirudin was from Sigma. All other chemicals were of the best commercially available grade.

Results

Effects of various growth factors on stimulation of AIB transport by glucagon

Fig. 1 shows that the inhibitory effect of EGF (Morin *et al.*, 1981) was not specific of this factor. PDGF, thrombin and FCS, which, like EGF, had no effect on basal transport in cultured hepatocytes, were also able to inhibit the stimulatory effect of glucagon (4–5-fold increase) on AIB transport. PDGF had a smaller effect (50% inhibition) at the arbitrary dose selected, presumably because we used a partially purified preparation (see the Materials and methods section). FCS was as potent as EGF (70–80% inhibition), whereas thrombin turned out to be the most powerful inhibitor (90%). Fig. 2 shows dose-response curves for FCS and thrombin. Inhibition of the glucagon effect was detected for low concentrations of serum (1%, v/v) and thrombin (0.1 unit/ml) and became nearly maximal with 12.5% FCS and 2.5 units of thrombin/ml. Surprisingly, the thrombin effect could not be prevented by a large excess of hirudin (results not shown), a protein reported to inhibit all the thrombin effects described so far (Markwardt, 1970).

As previously reported for EGF (Morin *et al.*,

Fig. 1. *Effect of various growth factors on glucagon stimulation of AIB transport*

Hepatocytes (24 h-monolayers) were incubated for 3 h without or with 0.1 μ M-glucagon, in the absence or presence of 1 nM-EGF, 0.1 μ M-PDGF, 10% (v/v) FCS or 1 unit of thrombin/ml. AIB (0.1 mM) transport was then measured over 15 min. Each bar is the mean $\pm$ S.E.M. of five experiments. Abbreviations: CONT, control; GLU, glucagon; THR, thrombin.

Fig. 2. Dose-response curves for FCS and thrombin effects on glucagon stimulation of AIB transport. Hepatocytes (24 h-monolayers) were incubated for 3 h without and with $0.1\mu\text{M}$ -glucagon in the absence or presence of various concentrations of FCS or thrombin. AIB transport was then measured over 15 min. Each point represents the mean of triplicate determinations.

1981), the other growth factors similarly inhibited the stimulatory effect of 8-bromo-cyclic AMP on AIB transport. Furthermore, the increase (4-fold) in amino acid transport occurring during the 3 h incubation in amino acid-free medium was not affected by growth factors (results not shown).

Effects of growth factors on the stimulation of AIB transport by various hormones

Besides glucagon (Pariza *et al.*, 1976; Morin *et al.*, 1981), insulin (Pariza *et al.*, 1977; Morin *et al.*, 1981) and catecholamines (Pariza *et al.*, 1977) have been shown to stimulate amino acid transport in cultured hepatocytes. Despite a common end point, there is presently no evidence that these different hormones act through the same mechanisms, which nonetheless all involve new protein synthesis. As illustrated in Fig. 3, FCS inhibited glucagon and phenylephrine effects but was totally ineffective in preventing insulin stimulation of AIB transport. Insulin effect was totally blocked by thrombin, which inhibited all hormonal effects equally well.

To rule out the possibility that the thrombin effect might result from its well known proteolytic properties (Markwardt, 1970), we carried out similar experiments with freshly isolated hepatocytes. In this system, none of the growth factors, including thrombin, was able to inhibit the stimulation of amino acid transport (results not shown). Thus, as suggested by the lack of inhibition of the thrombin effect by hirudin, proteolysis of hormones or receptors cannot account for the thrombin effect on hormone stimulation of transport in cultured hepatocytes.

In liver, catecholamines exert their known effects

Fig. 3. Effect of FCS and thrombin on hormonal stimulation of AIB transport.

Hepatocytes (24 h-monolayers) were exposed for 3 h to maximally effective concentrations of hormones ($0.1\mu\text{M}$ -glucagon and insulin, $50\mu\text{M}$ -phenylephrine) or growth factors [10% (v/v) FCS, 1 unit of thrombin/ml]. AIB transport was then measured over 15 min. Each bar represents the mean $\pm$ S.E.M. of five experiments. Abbreviations used, other than those defined previously: INS, insulin; PHE, phenylephrine.

Fig. 4. Inhibition by FCS of the α -adrenergic stimulation of AIB transport.

Conditions were as described in the legend to Fig. 1. The concentrations of effectors were: 10% (v/v) FCS; $10\mu\text{M}$ -phenylephrine; $50\mu\text{M}$ -DL-propranolol and phenoxybenzamine. Each bar is the mean $\pm$ S.E.M. of three experiments. Abbreviations used, other than those defined previously: PROPRA, propranolol; PHENOXY, phenoxybenzamine.

either through cyclic AMP-dependent (β -effect) or cyclic AMP-independent (α_1 -effect) mechanisms. Since growth factors were found to inhibit phenylephrine stimulation of transport it was important to determine which type of adrenergic mediation was involved. Fig. 4 shows that phenylephrine stimulation of AIB transport was insensitive to the specific β -blocker DL-propranolol; conversely, phenoxybenzamine (an α -blocker) totally prevented the phenylephrine effect. This observation, which is in agreement with previous studies (Pariza *et al.*, 1977), indicates that growth factors can inhibit the α -adrenergic effect of catecholamines on transport.

Inhibition by growth factors of hormonal stimulation of transport: dependence on culture conditions

Since no inhibition of hormonal stimulation of transport by growth factors could be detected in freshly isolated hepatocytes it became obvious that a change related to the culture conditions had occurred. As shown in Table 1 hepatocytes cultured for a short time (4h) were already sensitive to FCS, which largely prevented glucagon stimulation of AIB transport. The continuous presence of serum in culture medium was not required to maintain cell sensitivity to growth factors during culture. Indeed, the degree of inhibition of the glucagon effect on transport in 24h-cultured cells was analogous whether serum was absent or present in the culture medium (Table 1). It is likely, as observed for the synthesis of cytoskeleton proteins (Auberger & Le Cam, 1982), that cell attachment plays an important role in the appearance of the sensitivity of hepatocytes to growth factors.

Effect of FCS on the induction of tyrosine aminotransferase by glucagon and dexamethasone

To evaluate the specificity of the inhibitory effect of growth factors on hormonal stimulation of transport, we investigated the effect of FCS on the induction of tyrosine aminotransferase. In primary cultures of hepatocytes (24 h), enzyme activity was increased five times and seven times by glucagon

Fig. 5. Effect of FCS and thrombin on the induction of tyrosine aminotransferase by glucagon and dexamethasone

Hepatocytes (24 h-monolayers) were treated for 6 h without or with the effectors used alone or in combination before preparing 20000g supernatants from cell extracts (see the Materials and methods section) for tyrosine aminotransferase assays. The concentrations of effectors were: 10% (v/v) FCS, 0.1 μ M-glucagon, 0.1 mM-dexamethasone, 1 unit of thrombin/ml. Each bar is the mean $\pm$ S.E.M. of three experiments. Abbreviations used, other than those defined previously: DEX, dexamethasone.

and dexamethasone respectively, after a 6 h exposure of cells to hormones (Fig. 5). When FCS (10%) [or purified EGF (results not shown)] was added concomitantly to hormones, no inhibition of enzyme induction was observed (Fig. 5). In contrast, thrombin (1 unit/ml) largely prevented both glucagon and dexamethasone effects.

Table 1. Dependence on culture conditions of the inhibitory effect of FCS on glucagon stimulation of AIB transport
Hepatocytes were cultured for 4h or 24h as noted in the Table. Each set of dishes was then incubated for 3h in KRB medium, in the absence or presence of the effectors (10% FCS and 0.1 μ M-glucagon) before measuring AIB transport over 15 min. Each value is expressed as nmol of AIB transported per 15 min per mg of protein and is the mean of triplicate determinations.

Effectors	Culture conditions		
	4h with 10% FCS	4h with 10% FCS + 20h without FCS	24h with 10% FCS
None	0.38	0.57	0.48
Serum (10%)	0.37	0.56	0.50
Glucagon (0.1 μ M)	1.40	2.45	2.61
Glucagon (0.1 μ M) + serum (10%)	0.72	1.13	1.31

Effects of FCS and hormones on protein phosphorylation in cultured hepatocytes

Change in the degree of phosphorylation of proteins is a very early event that occurs on exposure of hepatocytes to hormones (Le Cam, 1982). Although it is not proven yet that phosphoproteins play a role in long-term effects (i.e., those involving protein synthesis), it was of interest to analyse the effects of growth factors on basal and hormone-stimulated protein phosphorylation. The set of ^{32}P -labelled proteins present in 24 h-cultured hepatocytes (Fig. 6) was very similar to that previously found in freshly isolated cells (Le Cam,

1982). The only significant change caused by growth factors, illustrated in Fig. 6 for FCS, was an increase in the labelling of a 34 000- M_r protein. This protein was found in ribosomes and identified as ribosomal protein S6, based on its acid solubility and its migration on two-dimensional gel electrophoresis. Insulin essentially increased the labelling of proteins with M_r 46 000, 34 000 and 22 000, whereas glucagon enhanced the ^{32}P content of many proteins, including the 46 000- and 34 000- M_r proteins. Phenylephrine alone had little effect. Regardless of the hormones used, none of their effects on protein phosphorylation was affected by the presence of FCS or other growth factors, including thrombin, at a concentration shown to maximally inhibit hormonal stimulation of transport.

Discussion

The present study confirmed and extended the original observation that EGF inhibits the stimulation of amino acid transport by glucagon in primary cultures of adult rat hepatocytes (Morin *et al.*, 1981). This was not a specific property of EGF since other growth factors, including PDGF, thrombin and FCS (presumably via EGF and PDGF), exerted the same inhibitory action. Such a result was somewhat anticipated, based on the common growth-promoting properties of all these factors in many cell types (Gospodarowicz & Moran, 1976; Pouyssegur *et al.*, 1982).

The inhibitory action of growth factors was not restricted to hormones acting through a cyclic AMP-dependent mechanism like glucagon. Indeed, catecholamine stimulation of transport occurring through the α -adrenergic mediation shown to involve change in intracellular Ca^{2+} concentration (Jenkinson *et al.*, 1978; Exton, 1979; Blackmore *et al.*, 1982) was similarly inhibited. The observation that the insulin effect on transport, presumably independent of any cyclic AMP change, was inhibited by thrombin but not by other growth factors might indicate that this hormone acts through a mechanism different, at least in one step, from that of glucagon and catecholamines. In addition, these results support the view that the various growth factors might exert their inhibitory effect at different levels in the cascade of events implicated in hormonal stimulation of transport. If one makes the assumption that appears reasonable, at least for glucagon, that a change in the degree of phosphorylation of endogenous proteins represents an early step in the complex pathway of the hormonal stimulation of amino acid transport, it seems that inhibition by growth factors involves rather late steps in the cascade of events that leads to the synthesis of new carrier proteins.

In most eukaryotic cells, including hepatocytes

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography of phosphoproteins present in cultured hepatocytes

Phosphorylation experiments were carried out as described in the Materials and methods section: 50 μg of proteins contained in sodium dodecyl sulphate/cell lysates from 24 h-cultured hepatocytes exposed to 10% (v/v) FCS, 0.1 μM -insulin, 0.1 μM -glucagon and 10 μM -phenylephrine (hormones used alone or in combination with FCS) were applied to each lane of the gel. An autoradiogram is presented. The arrows on the right indicate the major bands affected. Molecular-weight markers (left) were: myosin heavy chain, 200 000; β -galactosidase, 116 000; phosphorylase b, 94 000; serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soya-bean trypsin inhibitor, 20 000. Abbreviation: OR, origin.

(Kelley & Potter, 1979), besides the hormone-sensitive mechanism, amino acid transport can be regulated by substrates. The latter mechanism, called adaptive regulation, involves the synthesis of new carrier proteins when cells are incubated in the absence of amino acids (Guidotti *et al.*, 1978). The observation that growth factors failed to prevent the increase in the capacity of cultured hepatocytes to transport amino acids observed on a 3 h-incubation in amino acid-free medium suggests that hormones and substrates regulate this process at different levels in hepatocytes. Such an observation is in agreement with the previous finding that in cultured fibroblasts, insulin and amino acids affected different steps in the synthesis of carrier proteins (Guidotti *et al.*, 1974).

Hormonal control of tyrosine aminotransferase in hepatocytes has been shown to involve both RNA and protein synthesis (Ernest *et al.*, 1977). Recently, somatotropin was found to inhibit glucocorticoid induction of this enzyme in cultured hepatocytes (Shires & Hargrovel, 1982). In our study, none of the growth factors tested except thrombin prevented induction of the enzyme by glucagon or dexamethasone. Thus, inhibition of hormonal stimulation of transport appears to represent, to a certain extent, a specific effect for EGF, PDGF and FCS. That thrombin inhibited the effects on transport and enzyme induction, regardless of the hormone used, might suggest that it affected protein synthesis, either at the transcription or at the translation level. On the other hand, the other growth factors might exert their effect on transport at a different step, like the processing of newly synthesized carrier proteins. The hypothesis of such a post-translational control mechanism might be supported by the fact that growth factors did not prevent the hormone-mediated increase in phosphorylation of ribosomal protein S_6 . Indeed, based on the observation made in fibroblastic cell lines (Thomas *et al.*, 1979), where changes in degree of phosphorylation of S_6 were found to closely parallel those in the protein synthesis, an effect of growth factors at the translational level in our system might have involved inhibition of hormone effects on S_6 phosphorylation. This, however, remains speculative, but seems an attractive working hypothesis for future research.

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