Hormonal regulation of two urea-cycle enzymes in cultured foetal hepatocytes

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Foetal-rat hepatocytes were cultured in primary monolayer culture, and activity changes of argininosuccinate synthetase (ASS, EC 6.3.4.5) and argininosuccinase (ASL, EC 4.3.2.1) were followed under defined hormone conditions. In hormone-free medium, cultured cells maintained the enzyme activities at values equal to those of freshly isolated cells for at least 3 days. Continuous addition of dexamethasone produced the development of the two enzyme activities, but only after the first 20 h of culture. Under these conditions, urea production by the foetal hepatocytes was concomitantly increased in the culture medium. Pretreatment with dexamethasone for 20 h was sufficient to produce the development of ASL activity within the 2 following days. Introduced alone, glucagon induced an increase of ASL activity, but did not affect the ASS activity. The most powerful stimulation of ASS and ASL could be observed in cultured hepatocytes if glucagon and dexamethasone were added simultaneously or sequentially. These results indicated that the development of the receptor complex for the induction of urea-cycle enzymes appears early before birth and established that glucocorticoids amplify the glucagon stimulation of these enzyme activities during foetal life.

During development of the foetal rat liver, the appearance of a number of enzymes has been shown to result from hormone action (Jost & Picon, 1970; Greengard, 1970). Previous studies with whole foetal rat livers have indicated that four urea-cycle enzymes, namely carbamoyl phosphate synthetase (EC 6.3.4.16), ornithine transcarbamoylase (EC 2.1.3.3), argininosuccinate synthetase (ASS, EC 6.3.4.5) and arginase (EC 3.5.3.1), appeared in the liver of 17.5-day fetuses and could not develop without glucocorticosteroids (Gautier *et al.*, 1977). Argininosuccinate lyase (ASL, EC 4.3.2.1) was the only enzyme of the cycle that could not be affected by steroid addition or deprivation before term (Husson & Vaillant, 1979). In addition, ASL activity could be induced prematurely by administration of glucagon (or dibutyryl cyclic AMP) and by thyroxine *in utero* (Husson & Vaillant, 1978).

We also reported that, in corticosteroid-deprived fetuses, glucagon failed to promote ASL activity, although simultaneous administration of glucagon and cortisol increased the activity far more than the increases caused by a single hormone (Husson & Vaillant, 1982). These results showed that a synergistic interaction occurred between steroid and glucagon on ASL activity.

To obtain more definite information, we have used primary culture of foetal liver cells, which has been shown to be a very attractive model for studying development of some specific liver functions (Plas & Nunez, 1976; Leffert & Paul, 1973). A preliminary study has shown that, in such cells, the urea-cycle enzyme activities were stable without hormone addition in the culture medium (Husson *et al.*, 1982). In the present investigation, cultured hepatocytes derived from 17.5- or 18.5-day fetuses have been tested for their enzyme response to dexamethasone and glucagon. Several studies on urea-cycle enzymes in cultured adult cells had been made (Schimke, 1964; Gebhardt & Mecke, 1979; Lin *et al.*, 1982), but the development of urea-cycle enzyme activities in cultured foetal hepatocytes has never been investigated.

Materials and methods

Materials

Animals. Adult female rats of the Wistar strain were mated overnight and recognized as pregnant by a vaginal smear on the next morning. This was designated as day 0.5 of pregnancy. Livers from

17.5–18.5-day fetuses were used to prepare the primary cultures.

Chemicals. Glucagon was from Novo Therapeutisk Laboratorium (Copenhagen, Denmark). Dexamethasone, ornithine and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase and foetal-calf serum were purchased from Boehringer (Mannheim, Germany). The culture medium was a mixture of Eagle's Minimal Essential Medium and Medium 199 with Hanks salts, deficient in arginine, obtained in powder form from Eurobio (Paris, France). Glucose concentration was 5.5 mM. This medium was supplemented with 0.1 mM-ornithine, 5% or 10% (v/v) foetal-calf serum and Fungizone (0.25 µg/ml) and kanamycin (100 µg/ml), both from GIBCO (Grand Island, NY, U.S.A.).

All liquids were filtered through 0.22 µm-pore-diameter membranes (Millipore, London, U.K.) before use.

Methods

Culture of foetal rat hepatocytes. This was done by the method of Leffert & Paul (1972), with some modifications. For a typical culture, about 30 fetuses (17.5 or 18.5 days old) were delivered by caesarean section and decapitated immediately. The livers were collected aseptically, and cut into six to eight pieces, which were then pooled and washed twice in 10 mM-Hepes buffer, pH 7.5. The liver pieces were next incubated in a dissociation medium (Hepes buffer) with 0.03% collagenase for 10 min at 37°C, with gentle magnetic stirring. This enzymic digestion was performed twice, and the cell suspensions were then centrifuged for 1 min at 50 g in conical centrifuge tubes. The resulting pellets were pooled and washed three times in Hepes buffer, and packed cells were finally suspended in the growth medium. This selective medium, without arginine, prevented overgrowth of fibroblasts (Leffert & Paul, 1972) which cannot synthesize arginine from ornithine. Cell suspension (1 ml; approx. 2×10^6 cells/ml) was added to each 25 cm² tissue-culture flask with 4 ml of growth medium and incubated at 37°C in a humidified incubator under 5% CO₂ in air (CO₂ incubator; Forma Scientific, Marietta, OH, U.S.A.). The plating medium contained 10% foetal-calf serum to promote cell attachment.

To remove endogenous steroids, the foetal-calf serum was charcoal-treated before use. The serum was incubated for 20 h at 4°C in the presence of activated charcoal (10 mg/ml) and centrifuged (100 000 g for 30 min) at 4°C to eliminate the charcoal. This procedure removes all the endogenous corticosteroids, as measured by using a competitive protein-binding radioassay (Murphy, 1967).

Cell viability was tested by exclusion of the dye Trypan Blue, and the proportion of viable cells in final cell suspensions was always over 90%.

Fresh medium with 5% foetal-calf serum was given 5 h after plating and every 24 h thereafter. Additions of hormones were eventually performed 5 h after plating or when otherwise indicated, and enzyme activities were measured at the specified times thereafter. Samples of culture medium were removed at daily intervals and assayed for urea concentration, as described by Husson *et al.* (1981). Urea production is expressed as µmol/h per mg of total protein.

Preparation of cell extracts. After removal of culture medium, cell monolayers were washed twice with ice-cold Hepes buffer and the cells were harvested by scraping with a rubber policeman. The cell suspension was collected by centrifugation at 700 g for 5 min, and the pellet was homogenized in 0.1% *N*-cetyl-*NNN*-trimethylammonium bromide by freezing and thawing twice. All enzyme assays were made on fresh extracts.

Enzyme assay procedures. The homogenate was centrifuged at 4000 g for 15 min, and samples of the supernatant fraction were used for enzyme assays and protein determination (Lowry *et al.*, 1951). The activities of ASS and ASL were determined by the procedures of Ratner (1955). All enzyme activities are in units of µmol/h at 38°C, and specific activities are units/mg of protein. Results are expressed as means $\pm$ S.E.M., the numbers of observations being given in parentheses. Significance was determined by using a paired Student's *t* test.

Results and discussion

Freshly isolated cells became firmly attached to the flasks 5 h after inoculation, and within the first 24 h they formed a monolayer in which the cells were in close contact with neighbouring cells. ASS and ASL activities could be assayed reproducibly in homogenates prepared from freshly isolated cells or from cultured cells. In such extracts (Fig. 1), the two enzyme activities from 18.5-day foetal hepatocytes did not show any significant increase during 3 days in control medium. The rate of urea synthesis in culture medium measured each day also remained unaltered. As shown for other specific functions (Plas & Nunez, 1976; Leffert & Paul, 1973), these data demonstrate that the capacity for urea synthesis is maintained in cultured foetal hepatocytes and can be studied under various hormonal conditions.

Effect of dexamethasone

The requirements for the glucocorticoid inducer are shown in Fig. 1 and Table 1. When dexamethasone

Fig. 1. Effects of dexamethasone on the activities of ASS (a) and ASL (b) and on urea production (c) in 18.5-day foetal hepatocytes

Cells were maintained in control medium (●) or in the presence of 1 μM-dexamethasone (▲). Hormone was added 5 h after seeding, or 48 h thereafter. After the first 20 h of exposure to hormone, the medium was removed from some flasks and the cells were washed twice before reincubation in control medium. At the indicated time points, hepatocytes from each treatment were harvested and enzyme assays performed. Each point is the mean ± S.E.M. for at least three separate cultures.

Table 1. Effects of dexamethasone and glucagon on enzyme activities in cultured hepatocytes derived from 17.5-day foetuses

Cells were maintained during 3 days in control medium or in the presence of the specified compounds. Values are means ± S.E.M. for the numbers of separate cultures in parentheses.

Culture conditions	ASS (μmol/h per mg of protein)	ASL (μmol/h per mg of protein)
Control	0.48 ± 0.02 (4)	0.13 ± 0.05 (5)
Dexamethasone (1 μM)	1.00 ± 0.20 (6)	0.28 ± 0.02 (6)
Glucagon (10 nM)	0.49 ± 0.03 (4)	0.19 ± 0.06 (5)

methasone was continuously present in the culture medium, the activities of ASS (Fig. 1a) and ASL (Fig. 1b) were not affected until 24 h of culture, but then they increased progressively to day 3 of

treatment. Capacity for urea production (Fig. 1c) was also greatly enhanced in treated hepatocytes. We cannot establish a direct relationship between enhanced urea production and increased enzyme activities after dexamethasone treatment. Since glucocorticoids can produce protein degradation in liver cells (Gelehrter & Emanuel, 1973), we do not exclude that the increase in urea production in the culture medium with hormonal treatment would be mediated by increased concentrations of available amino acids.

When foetal cells were maintained in control medium for 48 h and dexamethasone was added for an additional 24 h, ASL activity was significantly enhanced on day 3, but not the ASS activity (Fig. 1). In this case, ASS activity did not rise significantly within 24 h, but its increase was significant compared with paired control cells 48 h later (results not shown). The results show that cultured hepatocytes

maintain their ability to respond to the steroid after 2 days in primary culture. The requirement for the continued presence of hormone in order to maintain induced enzyme activities was then determined. If dexamethasone was present in the medium during the first 20 h of culture only, ASL activity continued to develop to day 3 as if the hormone was continuously present (Fig. 1). In the same cultures, removal of dexamethasone resulted in cessation of ASS development. Thus the glucocorticoid inducer must be continuously present to permit development of the ASS activity.

When hepatocytes from 17.5-day foetuses were cultured for 3 days with dexamethasone (Table 1), the same induction was obtained of ASS and ASL activities. Although the glucocorticoid-receptor activity has been shown to be very low at this stage (Cake *et al.*, 1981), the competence to react to dexamethasone is acquired early in the foetal hepatocytes.

In general, our findings on foetal cells agree with the observations of Lin *et al.* (1982) on adult hepatocytes and show that glucocorticoids are also involved in the development of these enzyme activities before birth.

The effect of dexamethasone on increasing the specific activity of ASL in cultured foetal hepatocytes was found to be quite different from this after the administration of cortisol *in utero* (Husson &

Vaillant, 1979). Indeed, a lack of effect of cortisol on ASL activity *in utero* had been previously observed. The marked elevation of ASL activity after dexamethasone treatment *in vitro* suggests that the corticosteroid is an inducer of this activity, and it reflects that under conditions *in vivo* this effect was probably impaired. A suppressive phenomenon, such as the high plasma concentration of insulin (Felix *et al.*, 1969), may prevail *in utero* and may counteract the stimulation of enzyme activity by the corticosteroid (Räihä & Edkins, 1977; Ho *et al.*, 1981).

Effect of glucagon

When 18.5-day foetal hepatocytes were cultured continuously with 10 nM-glucagon (Fig. 2), the ASS activity was not affected at 3 days, but ASL activity was significantly increased over that of paired control hepatocytes. In 17.5-day foetal hepatocytes (Table 1), the same treatment during 3 days was without effect on the two enzyme activities. The lack of response at this precocious stage might be due to the lack of membrane receptors to glucagon, but this explanation seems unlikely, since glucagon could induce glycogenolysis in 15.5-day cultured foetal cells (Plas & Nunez, 1976). Another possible explanation for the above results is that foetal adrenals start to secrete steroids on day 17.5, and

Fig. 2. Time course of effect of glucagon on (a) ASS and (b) ASL activities and potentiation of glucagon action by dexamethasone

Foetal hepatocytes (18.5 days) were cultured for 3 days in control medium (●), with 10 nM-glucagon (■), or with dexamethasone (1 μ M) and glucagon (10 nM) (△). In some flasks, cells were cultured with dexamethasone (●—▲) for the first 20 h, then they were washed twice and glucagon was added for the 2 following days. At the indicated time points, cells were harvested and enzyme assays performed. Each value is the mean of at least three separate cultures; the S.E.M. never exceeded 10%.

the cells that are cultured at this stage have not yet undergone the influence of corticosteroids and therefore are not mature enough to respond to glucagon.

Combined effects of dexamethasone and glucagon

When dexamethasone and glucagon were present simultaneously in the culture medium, the increases in ASS and ASL activities exceeded the increase caused by each hormone alone on day 3 of culture (Fig. 2). Particularly, the response of ASS activity was synergistic, since both effectors gave a greater than additive increase. The response of ASL activity was additive, since it was almost equal to the sum of the increases observed in the presence of either glucagon or dexamethasone alone. The fact that glucagon stimulated ASL activity without a requirement for dexamethasone, whereas its effect on ASS was dexamethasone-dependent, suggests a different regulation of the latter enzyme.

When dexamethasone and glucagon were added successively in the culture medium (Fig. 2), ASS activity increased far more than with glucagon alone, but the increase was not as high as this after the simultaneous addition of both hormones. In this case, the increase in ASL activity was similar to that after the simultaneous addition of the two compounds. These results show that the two hormones act sequentially by different mechanisms and suggest, as proposed for tyrosine aminotransferase induction (Ernest *et al.*, 1977), that dexamethasone might induce the synthesis of an intracellular factor (mRNA?) which permits the glucagon effect on the enzyme activities of the foetal cells.

The present experiments on urea-cycle-enzyme inducibility in such a system *in vitro* were performed in view of the observations in adult rat hepatocytes (Gebhart & Mecke, 1979; Lin *et al.*, 1982) that dexamethasone and glucagon were effective inducers of these enzyme activities. The results clearly indicated that the receptor complex for the induction of urea-cycle enzymes is acquired early and sequentially in foetal life and proved that

these enzymes are good markers for study of the terminal differentiation of foetal hepatocytes.

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