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IN VITRO TECHNIQUES FOR STUDIES ON PITUITARY REGULATION OF RAT LIVER ENZYMES

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SUMMARY

Techniques have been developed for studies on synthesis and release of the novel pituitary hormone, feminotropin, from pituitary cells in primary monolayer culture. Feminotropin activity can be estimated in primary monolayer cultures of liver cells. Both kinds of cells are kept in well defined media containing only 1–2% serum in order to facilitate studies of hormonal influence on the cells. The pituitary cells produce hormones, mainly prolactin, for more than 4 weeks. When kept in [^{3}H]-leucine-containing medium, mainly labelled peptides and/or proteins with isoelectric points between 4 and 7 are released. When suitable amounts of pituitary cells or when pituitary cell medium are transferred to cultures of hepatocytes the pattern of steroid metabolism in the hepatocytes is changed in a feminine direction.

INTRODUCTION

Sex differences in hepatic steroid metabolism in rats are completely dependent on the presence of an intact hypothalamo-pituitary axis [1–6]. Hypophysectomy of female rats results in a partial masculinization of liver metabolism whereas identically treated castrated male rats retain their pattern of liver metabolism [1–4]. Transplantation of a pituitary gland of male or female origin under the kidney capsule of a hypophysectomized rat of either sex results in feminization of hepatic steroid metabolism [2, 4]. Based on these and other experiments we have postulated the secretion of a "feminizing factor" or "feminotropin" from the female and the autonomous pituitary, which is different from the established hypophyseal hormones and that feminizes a basically male type of liver steroid metabolism [1, 3–8]. In order to investigate the nature and formation of feminotropin it has been essential to establish tissue culture techniques for cultivation of normal pituitary and liver cells. This paper describes the methods used in preparation and culture of isolated pituitary and liver cells and also describes results from some recent experiments performed with these systems.

MATERIALS AND METHODS

Isolation and culture of pituitary cells. Pituitary cells were prepared as described by Vale *et al.*[9] with the modification that the cells were dispersed by an initial incubation of the quartered pituitary tissue with 0.1% (v/v) collegenase Type I (Sigma) for 30 min followed by an additional incubation for 30 min with 0.5% (v/v) Viokase (Gibco). The cells were then washed and suspended in Leibowitz L-15 medium supplemented with 2% (v/v) male rat serum, 10 mM glucose, 1 mM disodiumsuccinate and 60 mg/100 ml of benzylpenicillin; 1–2 × 10^5 cells in 1 ml medium were added to each 35 mm plastic Petri dish (Nunco, Denmark). The pituitary cells formed continuous monolayers for periods longer than four weeks.

Isolation and culture of liver cells. The isolation procedure was a modification of the procedure described by Seglen[10, 11]. Sprague–Dawley rats 8–9 weeks old were used. After inducing ether anesthesia 500-1000 IU of heparin was given intravenously. A calcium-free buffer (144 mM NaCl–7.0 mM KCl–10.1 mM HEPES–11 mM glucose–5.1 mM sodium pyruvate–5.8 mM sodium fumarate–4.7 mM sodium glutamate, adjusted to pH 7.4 with NaOH) was infused via the portal vein and drained from a cut in the inferior vena cava. After 5 min a collagenase buffer (Eagle's minimum essential medium supplemented with 2.0 mM $CaCl_2$, 26.6 mM HEPES, 26.4 mM TES, 31.8 mM Tricine and 50 mg/100 ml of Sigma collagenase Type I, pH 7.4) was infused. The solution was taken out from a second cannula in the inferior vena cava and recycled for 10 min. The infusion rate was about 50 ml/min and the temperature of the solution was 37°C.

The liver was then removed and cooled in ice-cold washing buffer (Eagle's minimum essential medium supplemented with 26.6 mM HEPES, 26.4 mM TES and 31.8 mM Tricine, pH 7.4). The remaining connective tissue was disrupted mechanically and the cells released into the washing buffer. Connective tissue and remaining liver fragments were separated by filtration through cotton gauze. The cell suspension was centrifuged at 100 *g* for 2 min. The cells were resuspended and washed two to three times and were finally suspended in culture medium (Leibowitz L-15 medium supplemented with 1% (v/v) male rat serum, 10 mM ammonium acetate, 10 mM glucose, 1 mM disodium succinate and 60 mg/100 ml benzylpenicillin. The yield of liver cells was about 2–5 × 10^8 cells per liver. The dispersed cells were kept in 35 mm Petri dishes (5 × 10^5 cells in 1 ml of medium) and

the medium was changed after one day. After a week, most of the liver cells still excluded Trypan blue and attached to the Petri dish.

It should be emphasized that the medium used for cultivation of both hepatocytes and pituitary cells contained much less serum (1 and 2%, respectively) than utilized by most workers. A more highly defined medium like the one used in the present investigation should facilitate studies on hormonal control of cellular metabolism. In addition, homologous serum was used in the present study in contrast to the general use of heterologous serum (calf serum, horse serum) in cell culture. It is reasonable to assume that the use of homologous serum should create a more physiological milieu to the cells than when heterologous serum is included in the tissue culture medium.

Enzyme assays. Methods to assay activities of steroid-converting enzymes in rat hepatocytes have been described in detail previously [12].

Radioimmunoassay of prolactin was carried out as described previously [8].

RESULTS AND DISCUSSION

Synthesis and release of peptides from pituitary cells in culture

Pituitary cells in monolayer culture have been shown to release prolactin and somatotropin [9]. In the present study, radioimmunassay of the cell culture medium collected from pituitary cells showed that these cells continued to produce prolactin and also respond to TRH, by a 10-fold increase in prolactin production, after more than 3–4 weeks in culture. In order to obtain a total estimation of the peptide-synthesizing capacity of the pituicytes, these were kept in [^{3}H]-leucine-containing medium for 48 h and the radioactive peptides released into the medium were analyzed by isoelectric focusing. As can be seen in Fig. 1, a reproducible pattern of four major peaks with pI-values of 4.63, 5.06, 5.63 and 6.12 was recorded. Radioimmunoassay revealed that the peptide with the lowest pI reacted identically to prolactin.

Effects of products of pituitary cells in culture on steroid metabolism of liver cells in culture

As established from experiments *in vivo*, feminization of hepatic steroid metabolism includes a decrease in the apparent V_{max} value of the 16α-hydroxylase active on 4-androstene-3,17-dione as well as an increase in the apparent V_{max} value and a decrease in the apparent K_m value for the 5α-reductase active on the same steroid substrate [13]. Therefore, the ratio between 5α-reduction and 16α-hydroxylation (5α/16α ratio) may be taken as an index of feminization of hepatic steroid metabolism [12].

Hepatic steroid metabolism was preserved relatively well in the hepatocytes kept in culture. As shown in Fig. 2, the apparent V_{max} values for the 5α-reductase and 16α-hydroxylase enzymes decrease by about 50% when the hepatocytes were kept in culture for 2–3 days. No change was noted in the apparent K_m of the enzymes.

When hepatocytes were kept in culture together with pituitary cells, an initial dose-dependent rise in the 5α/16α ratio was followed by a dose-dependent decrease in the ratio (Table 1). A similar type of dose-dependent metabolic change was seen following addition of various amounts of pituitary granules to tissue cultures of hepatocytes [12] and the results may be interpreted to reflect an initial feminization of liver

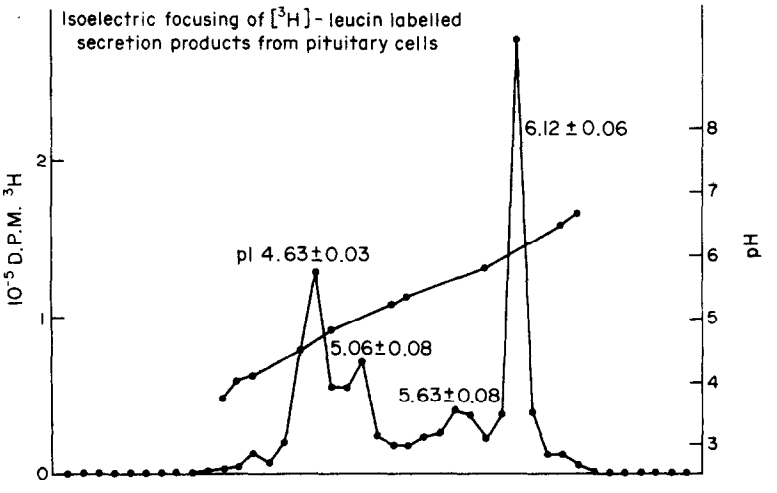

Fig. 1. Isoelectric focusing of [^{3}H]-leucine-containing peptides from pituitary cells in tissue culture. Monolayers of pituitary cells kept in culture for 2–3 weeks were kept in male-rat-serum-fortified Eagle's minimum essential medium, containing 10 μCi of [^{3}H]-leucine (specific radioactivity, 1.09 Ci/mmol) per ml, for 48 h. The medium was desalted by chromatography on Sephadex G-25 (equilibrated in 10 mM Tris–HCl, pH 7.4, 10 mM KCl and 1 mM EDTA) and analysed by isoelectric focusing. Electrofocusing was performed in 6 ml columns [14] stabilized by a 15–60% (w/v) sucrose gradient using Ampholine® of pH 4–8. A maximum of 0.2 W was applied for 16 h at 4°C. Measurements of pH were performed at the same temperature. The oblique line connects the measured pH values. The pI values were calculated from three experiments.

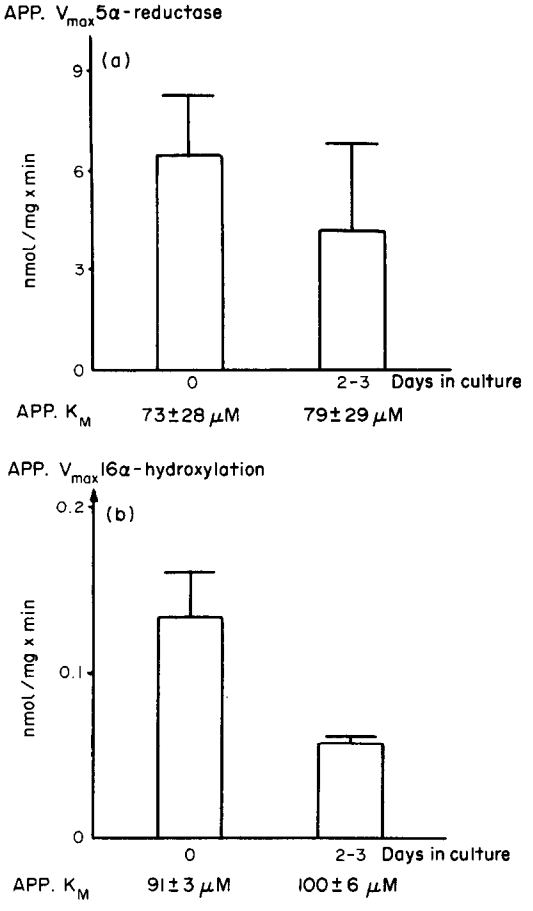

Fig. 2. 5α-Reduction and 16α-hydroxylation of 4-androstene-3,17-dione in hepatocytes immediately after isolation (0) and after 2–3 days in culture. The apparent V_{max} and K_m values were calculated from Lineweaver–Burk plots. 1 S.D. is indicated on top of the bars. The decrease in apparent V_{max} values for the 5α-reductase was not significant whereas the decrease in the corresponding value for the 16α-hydroxylase was significant ($P < 0.001$).

cell metabolism caused by feminotropin and a following loss of feminotropin effects due to increased interference by other hormones.

In order to purify feminotropin from other peptides released from pituitary cells, medium obtained from pituicytes in culture was fractionated by isoelectric focusing following gel-filtration on Sephadex G-25. Results from additions of pituitary cell medium components with pI-values between 4.4 and 6.5 and between 7.2 and 8.9 to hepatocytes in culture are shown in Fig. 3. A feminization of liver cell metabolism (*i.e.* an increased 5α/16α ratio) was seen following exposure of liver cells to the components of pituitary cell medium with low pI values. We are presently investigating whether one or a combination of the four [^{3}H]-leucine-labelled peptides produced by the pituitary cells (*cf.* Fig. 1) is responsible for the observed feminotropin activity. No feminotropin activity was detected in the fraction of the pituitary cell medium with high pI values. This may indicate that the previously described feminotropin activity with a pI value of 8.3 present in pituitary cell homogenates [6] and assayed with tissue cultured hepatoma cells [7] possibly is a precursor of feminotropin.

Table 1. Changes in the 5α/16α ratio (see text) of rat hepatocytes in tissue co-culture with increasing amounts of rat pituitary cells. Hepatocytes and pituitary cells were mixed one day after isolation and kept in co-culture in hepatocyte medium for two days. The concentration of hepatocytes was 5×10^5 cells/ml of medium. No metabolism of 4-androstene-3,17-dione was detected when the steroid was incubated with pituitary cell homogenates under the same conditions as when incubated with homogenates of mixed hepatocytes and pituitary cells

Amount of pituitary cells/ml of tissue culture medium	5α/16α ratio (%)
0	100* ± 16
2,500	141 ± 40
5,000	197 ± 80†
10,000	182 ± 17‡
20,000	97 ± 1

* Absolute ratio in control experiment was 3.04.
† $P < 0.05$ when compared to control.
‡ $P < 0.01$ when compared to control.

In conclusion, methods are presented for isolation and cultivation of rat pituitary cells and rat hepatocytes. These methods have proved to be of value in studies of feminotropin release from pituitary cells

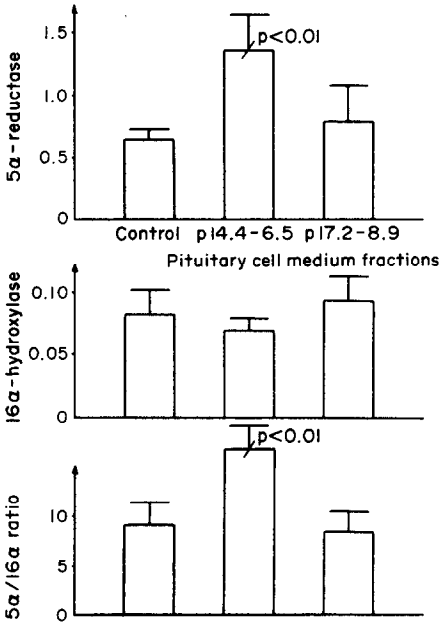

Fig. 3. 5α-Reduction and 16α-hydroxylation of 4-androstene-3,17-dione and 5α/16α ratio in hepatocytes in culture after addition of acid (pI 4.4–6.5) or alkaline (pI 7.2–8.9) components of medium from tissue cultured pituitary cells. Medium from pituitary cells in 3-week-old cultures was desalted by chromatography on Sephadex G-25 and fractionated by isoelectric focusing as described in the legend to Fig. 1 but using Ampholine® in the range of pH 3.5–10. Before addition to the hepatocytes the fractions obtained after electrofocusing were chromatographed on Sephadex G-25 columns (equilibrated in phosphate-buffered physiological saline, pH 7.4). The amount added per Petri dish corresponded to 0.3 ml of 48 h medium. Hepatocytes were harvested 18 h following addition of pituitary extracts.

and work is now in progress in our laboratory to characterize feminotropin and to study the factors regulating its biosynthesis and release.

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DISCUSSION

Friesen. I wonder if we are being confused by the term feminotropin. What is the most conclusive and compelling reason why feminotropin is not the same as prolactin?

Stenberg. Prolactin has been administered both *in vivo* and *in vitro* to hepatoma cells and hepatocytes in monolayer culture. We cannot obtain any feminization as measured by several sexually differentiated enzyme activities active on various steroid substrates either *in vivo* or *in vitro* with prolactin.

Friesen. Does antiserum to prolactin inhibit or combine with any of the radioactively labelled proteins which you showed—I think it was on your last slide—or does that block any of the effects of feminotropin?

Stenberg. That is an interesting question. We haven't any results from radio-immunological assays on these tritiated peptides yet, but experiments are in progress to elucidate these aspects.

Gustafsson. To summarize the evidence why feminotropin is not identical to prolactin, as was also indicated by Dr. Naftolin in his comments to Dr. De Moor's paper:

1. When prolactin is given to male and female rats, we do not see any effects on steroid metabolism either in castrated or in intact male or female rats (Gustafsson J.-Å. and Stenberg Å.: Influence of prolactin on the metabolism of steroid hormones in rat liver and adrenals. *Acta endocr., Copenh.* **78** (1975) 545–553.

2. We have given CB-154 and both in male and female rats prolactin levels are increased although liver enzyme activities are not affected.

3. We have administered perphenazine which results in increased levels of prolactin although no effects are seen on liver metabolism.

4. There is no reason to think that prolactin should be involved in regulating sex differences in liver metabolism since prolactin levels in male and female rats are about the same. It may be argued that even if prolactin levels are identical in male and female rats there may be sexually different responses for prolactin in the liver, i.e. prolactin receptors may be present in livers from female rats but not in livers from male rats. If this is the case, you have to postulate the existence of another pituitary factor that induces the prolactin receptors.

5. We have tried to repeat the results on liver membrane receptors of Dr. Friesen and Dr. Posner and have been able to reproduce them with ovine prolactin and also with human placental lactogen, with much greater binding of ovine prolactin and human placental lactogen in female rats than in male rats. However, when we iodinate rat prolactin given by NIH with the Bolton–Hunter method, which is thought to be a mild method, we cannot find any sex difference in prolactin binding in rat liver. In fact, iodinated rat prolactin binds to a very small extent to rat liver membranes.

6. Our *in vitro* results presented by Dr. Stenberg quite clearly indicate that rat prolactin is not involved, since when Dr. Stenberg adds NIH rat prolactin to isolated hepatocytes in culture he in fact observes a decrease in the 5α reductase level whereas feminotropin increases the 5α reductase activity. So, in summary, I think we have got quite conclusive indications that feminotropin is not identical to rat prolactin.

Posner. I think there are a lot of questions raised by observations on feminotropin that cannot be easily answered by postulating that it is identical to prolactin. Whether in fact it's going to turn out to be separate or not is still a matter of further work of course. The observation that the specific binding of [^{125}I]-rat-prolactin to membranes is minimal in your hands deserves further comment. An important caution is that negative data may not mean anything. We have also observed minimal binding of [^{125}I]-rat-prolactin to various membrane preparations in which unlabelled rat prolactin very effectively competed with [^{125}I]-oPRL or hGH for binding. The ability to see binding of labelled rat prolactin may simply reflect problems in iodinating the material and in retaining a reasonably intact hormone after the usual iodination procedures.

The essential point that you are making really is that you see *in vitro* effects with whole pituitary extracts that you do not see with pure prolactin. Have you used prolactin other than rat prolactin *in vitro*? Have you used combinations of hormones other than prolactin? It may well be that a prolactin effect is crucial but it requires synergism with something else.

Gustafsson. If we use various combinations of hormones, we do not see any effects on feminization in the hepatoma cell system. Other prolactins than the rat prolactin do not give any effects either. As Dr. Stenberg indicated, a crude pituitary extract does not give any effects on the isolated liver cells although feminization occurs in the hepatoma cell system. However, with purified preparations of the pituitary extract, we see feminization in the liver cell system as well.

Solomon. You have two enzymatic activities in the liver, reduction and hydroxylation. Can you show increases for instance in the *P*-450 hydroxylating pattern? Have you looked at that side of life?

Gustafsson. We are very interested in that aspect. If you look at cytochrome *P*-450 levels in rat liver there are certain sexual differences (slightly higher levels in males) but they are not so high as you would expect. However, recent work has shown that cytochrome *P*-450 is not a single enzyme but is composed by several different, chemically very related species (Gustafsson J.-Å. and Ingelman-Sundberg M.: Multiple forms of cytochrome *P*-450 in rat liver microsomes. Separation and some properties of different hydroxylases active on free and sulfoconjugated steroids. *Eur. J. Biochem.* **64** (1976) 35–43). So the reason why the sex difference with regard to the *P*-450 concentration in liver is not so apparent as with the different enzyme activities we measure is probably because the *P*-450 concentration only reflects the sum of all hepatic hydroxylase activities and this sum may well be the same in male and female rats. In order to perform any kind of meaningful studies on regulation of hydroxylase activities it is necessary to measure single hydroxylase activities. The hydroxylase activities we measure are all cytochrome *P*-450-dependent because they are all inhibited by carbon monoxide and also by other agents that inhibit the catalytic activity of cytochrome *P*-450.

Posner. I am sorry I made a mistake. I thought you talked about an effect of whole pituitary extract on the hepatoma cells. Is it whole pituitary extract or is it a fraction of the whole pituitary extract.

Gustafsson. Both with the crude extract and with the purified extract.

Posner. With the hepatoma cells only, not with the hepatocytes?

Gustafsson. With the hepatocytes you need purer preparations.

Posner. But you do see an inductive effect with the hepatocytes?

Gustafsson. Yes, the overall results from the hepatoma and the hepatocyte cell systems are in good agreement. The reason why we use the hepatoma cells is that these are easier to work with than isolated hepatocytes. However, we have considered it essential to perform similar studies also with hepatocytes to ascertain that the effects we see of pituitary extracts on the 5α-reductase are not phenomena restricted to tumour cells.

Solomon. Do your hepatocytes make albumin?

Gustafsson. Yes.