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EFFECTS OF PROGESTIN ANTAGONISTS, GLUCOCORTICOIDS AND ESTROGEN ON PROGESTERONE-INDUCED PROTEIN SECRETED BY RABBIT ENDOMETRIAL STROMAL CELLS IN CULTURE

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Summary—Progesterone enhances the synthesis of a 42 kDa protein secreted by rabbit endometrial stromal cells in primary culture. The duration of that response, the effects of estrogen and the inhibitory ability of antiprogestin steroid analogs, RU486, ZK98.299 and ZK98.734, were tested. Although there was a progressive decrease in the amount of the 42 kDa protein synthesized during a 6-day culture period, progesterone stimulated its rate of synthesis >2-fold throughout that period. The addition of estrogen did not prevent the progressive decrease in the amount of the protein synthesized, nor did it enhance the progesterone effect when the culture medium contained phenol red. Estrogen alone did slightly induce 42 kDa protein synthesis by cells grown in phenol red-free medium, and the progesterone response was accentuated to the same degree. When present in a concentration that was 100-fold that of the progesterone, RU486, ZK98.299 and ZK98.734 each abolished stimulation. This antagonistic effect was overcome by addition of an equimolar concentration of progesterone. Deoxycorticosterone (DOC) also stimulated 42 kDa protein synthesis. The antiprogestins blocked this stimulatory effect, even when both steroids were in equimolar concentrations. There was no difference in the ability of ZK98.299 or ZK98.734 to block DOC stimulation, even though ZK98.734 exhibits no antiglucocorticoid activity [*J. Steroid Biochem.* **25** (1986) 835]. Therefore, it is likely that the DOC effect is mediated by the progesterone receptor system. These studies indicate that enhanced synthesis of the 42 kDa protein represents a progesterone receptor mediated event and that the cell culture system described can be used as a bioassay for determination of antiprogestin activity.

INTRODUCTION

Progestins are known to play a role in cellular differentiation in the reproductive tract and mammary glands. Through studies performed on chick oviduct cells and mouse mammary tumor virus infected cells there have been great strides made in our understanding of steroid hormone action at the molecular level [1]. Although there are reports of the negative effects of progesterone (P) on P receptor and estrogen receptor content of cells in culture [2, 3], there exist few culture systems in which the stimulatory action of progesterone can be easily and routinely assessed in mammalian cells [4]. Breast cancer cells have proved an important model for studying estrogen and progestin action but the endpoints of a positive effect of P are often cumbersome, requiring large numbers of cells and laborious biochemical procedures [5–7]. Progestins also stimulate human endometrial cells in primary culture [8–10] but the source of such cells is sporadic and the hormonal milieu from which they are derived cannot be experimentally controlled. There has yet to be a description of an endometrial cell line that can be stimulated by P in culture. Thus, reliance on classical *in vivo* bioassays has persisted in the development of new progestin and antiprogestin steroid analogs [11].

Rabbit endometrial stromal cells in primary culture respond to P stimulation by increased production and secretion of a protein that migrates as 42 kDa in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions [12]. Enhanced synthesis of this protein was shown to be dose-dependent and specific to P. Therefore, rabbit P-enhanced

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42 kDa protein could be utilized as the endpoint in a bioassay system for P or antiP activities.

In the present study, the duration of the P response and the possible modification of this response by estrogen were examined. Additional studies on the specificity of the response were performed and the effects of antiprogestin/antiglucocorticoid steroid analogs were tested. The effect of pretreatment of the animals from which the cells were derived was also examined.

EXPERIMENTAL

Materials

Adult, nulliparous New Zealand rabbits (3–3.5 kg) from a local supplier were used as the source of endometrial cells. The following steroids were purchased from Steraloids (Wilton, NH): progesterone (P), estradiol-17β (E_2), deoxycorticosterone (DOC), dexamethasone and dihydrotestosterone. Antiprogestin/antiglucocorticoid steroid analogs ZK98.299 (ZK299) and ZK98.734 (ZK734) were gifts from Schering AG (Berlin, Germany) and RU486 was a gift of Roussel UCLAF (Romainville, France). Radiolabeled methionine ([^{35}S]-methionine, 1500 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Ham's F12, buffered salt solutions and antibiotic solutions used in cell culture were purchased from GIBCO (Grand Island, NY). Proteases used in cell dissociations were Trypsin 1:250 (Difco Laboratories, Detroit, MI) and collagenase (GIBCO). Phenol red-free medium, DME–Ham's F12 (1:1, v/v); Cohn's fraction V bovine serum albumin (BSA) and soybean trypsin inhibitor were from Sigma (St Louis, MO). Electrophoresis chemicals were from Bio-Rad (Richmond, CA). Film for fluorography, X-Omat AR, was purchased from Kodak (Rochester, NY). Fluorographic solution (Amplify) and scintillation fluid were purchased from Amersham.

Tissue dissociation and cell culture

Stromal cells were harvested from uteri by serial enzymatic digestions as follows: uterine horns were cut into approx. 2-cm lengths, everted and placed in a flask with 15 ml of 1% trypsin, 0.25% collagenase in calcium and magnesium free phosphate buffered saline (PBS-CMF). The flask was incubated at 37°C with vigorous shaking for 20 min. The enzyme solution containing a cell suspension was removed to a centrifuge tube and 10 ml fresh enzyme solution was added to the flask. The flask was again incubated with shaking; the process was repeated until 6 separate cell fractions were obtained. The cells from each fraction were pelleted by centrifugation and resuspended in 1% soybean trypsin inhibitor in Hank's balanced salt solution (HBSS). The first fraction contained mostly epithelial cells and it was not used in the experiments described. Cells from the other 5 fractions were combined, washed twice in HBSS and resuspended in culture medium.

The cells were cultured in 24 well plates in the following medium: Ham's F12, containing antibiotics (penicillin, streptomycin and neomycin), 10 μg/ml insulin, 5 μg/ml transferrin and 1 mg/ml BSA. The uterus of 1 animal yielded 50–100 million cells; these were plated at 0.5×10^6 cells/0.5 ml/well. Two days after plating, cells were washed with HBSS and fresh culture medium without BSA was added. To this medium was added: [^{35}S]methionine (25 μCi/ml final concentration) and steroid hormones (dissolved in ethanol, final concentration of ethanol 0.1%) or vehicle, 3–4 wells/treatment. In most experiments, the medium was harvested from all wells 24 h after addition of hormones and labeled methionine; the wells were usually confluent at this point.

In a set of experiments in which the duration of the hormone response was tested, hormones (P, E_2 or E_2 + P) were added after the initial wash of the plates on the second day of culture and [^{35}S]methionine was added either at that time or on the 3 successive days thereafter. Media was collected 24 h after addition of the labeled methionine, i.e. at 24, 48, 72 or 96 h after P was added. In another experiment examining the effect of E_2, cells were cultured in DME–F12 without phenol red as the basal medium. After washing the cells, E_2 was added at 10^{-11} M or 10^{-9} M and 24 h later 10^{-8} M P was added along with [^{35}S]methionine. Media samples were collected 24 h later.

Protein electrophoresis

In earlier studies [12] trichloracetic acid (TCA) precipitation had been used to normalize the amount of sample applied to each gel lane; however, it was found that the amount of total TCA precipitable protein in the medium was dependent upon cell number and it was not increased by P. Likewise, intracellular TCA precipitable and TCA soluble [^{35}S]methionine

was not discernibly affected by P treatment. Since the culture wells were confluent at the time of testing, the TCA precipitation procedure was omitted and equal amounts of media samples were analyzed by SDS–PAGE with fluorography. Samples (10 μl) were electrophoresed on 7–15% polyacrylamide gradient minigels (7.5 × 10 cm). The gels were fixed in methanol/acetic acid (50/8%) for 1 h and soaked in Amplify before drying. Fluorographs were made by exposing X-Omat AR film to the gel at −70°C for 3–5 days. The response to hormone was measured by densitometric analysis of the fluorographs using a Bio-Rad model 620 video densitometer. The amount of newly synthesized 42 kDa protein was expressed as a percentage of the total [^{35}S]methionine incorporation (total optical density of all bands) and thus any variation in total protein synthesis would not falsely register as an effect on 42 kDa protein.

In vivo pretreatment

In a preliminary experiment, 8 adult (3–3.5 kg) rabbits were ovariectomized under ketamine–acepromazine anesthesia. Two weeks later the rabbits were injected with 4 daily doses of E_2 (1 mg), P (5 mg), E_2 + P (1 and 5 mg, respectively) or vehicle (sesame oil); 20 h after the last injection the animals were sacrificed and their uterine horns were chopped into several pieces of approx. 0.5–1 cm cubed. These pieces were placed in 18 mm diameter culture wells with Ham's F12 containing 100 μCi/ml [^{35}S]methionine with or without 10^{-8} M P.

In a similar experiment, intact, nulliparous, adult animals were treated with 5 mg P, others were treated with vehicle; 20 h later they were sacrificed and their uterine horns were placed in explant culture as above, with or without 10^{-8} M P.

Statistical analysis

All data were subjected to analysis of variance using a statistical program (Statview, Brain-Power, Inc., Calabasas, CA) on an Apple Macintosh SE microcomputer (Apple, Cupertino, CA). For experiments in which results of a single set of cultures were analyzed, differences between individual treatment means and the mean control value (vehicle treatment) were analyzed by a least squares difference (Fisher PLSD). When the treatment replicates from separate experiments were included in the means, differences among individual means were analyzed by the Scheffe F-test.

RESULTS

There was continuous stimulation of 42 kDa protein synthesis when P was present in the culture medium for 4 days, during the 3rd–6th days of culture [Fig. 1(A)]. Although the amount of protein synthesized decreased progressively during that culture period, the relative increase produced by P stimulation remained constant. When phenol red was present in the medium, the addition of E_2 during the 4 days of P treatment neither increased the response nor

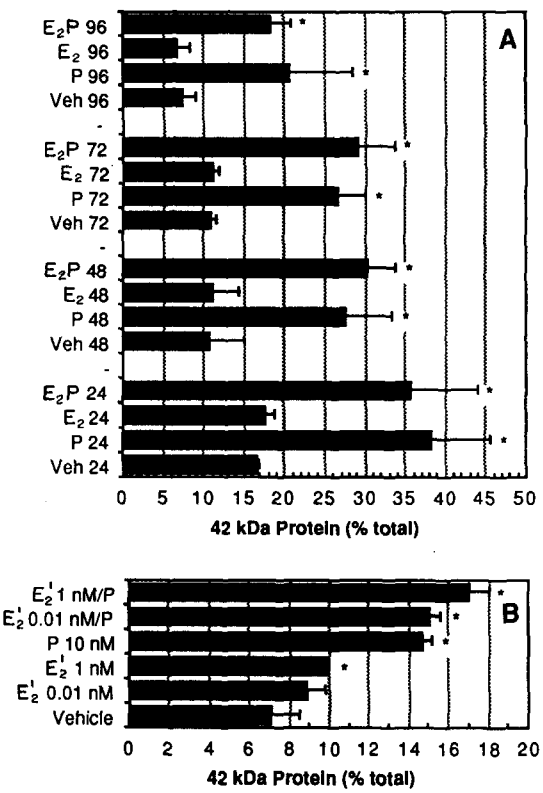

Fig. 1. Duration of P responsiveness and the effect of estrogen. (A) Cells were cultured for 2 days before hormone was added. At t = 0 h, 10^{-8} M P, 10^{-9} M E_2 or both hormones (E_2P), were added to 4 groups of culture wells. Media samples were collected at 24, 48, 72 or 96 h; [^{35}S]methionine was added to the cultures 24 h before media collection. (B) Cells were cultured for 2 days in phenol red-free medium and cells were treated by addition of E_2 at 10^{-11} M (0.01 nM) or 10^{-9} M (1 nM) or vehicle. The next day P was added at 10^{-8} M (10 nM) to culture wells that already contained E_2 (E_2P) or to those that were not estrogen primed (P). Samples of medium proteins were separated by SDS–PAGE, detected by fluorography, and analyzed densitometrically. The concentration of the 42 kDa protein is expressed as the percentage of total radiolabeled proteins in the medium. Mean ± SEM is presented; *treatment means that differed from the vehicle treatment mean, $P < 0.05$.

attenuated the progressive decrease in overall synthesis of the 42 kDa protein [Fig. 1(A)]. However, when the cells were cultured in a phenol red-free medium there was a slight increase in the synthesis of the protein due to addition of E_2 alone and the effect of P was enhanced to the same degree [Fig. 1(B)].

The antiprogestin/antiglucocorticoid steroid analogs, ZK299, ZK734 and RU486 were equally effective in blocking P stimulation of 42 kDa protein synthesis [Fig. 2(A)]. When P and antagonists were added to the cultures in equimolar concentrations (10^{-6} M) the antagonistic effect was overcome [Fig. 2(B)].

Testing the specificity of the hormonal response revealed that DOC, at 10 nM or 1 μM, was approx. 50% as effective in stimulating 42 kDa protein synthesis as the same concentrations of P [Fig. 2(A, B)]. Dihydrotestosterone had no effect when present alone or in combination with P (data not shown). The synthetic glucocorticoid, dexamethasone, would occasionally stimulate 42 kDa protein when the steroid was present at 1 μM but this varied from an insignificant amount to about 60% above control level among different experiments (data not shown). The steroid antagonists were effective in blocking stimulation by DOC [Fig. 2(A)]. However, unlike P, an equimolar concentration of DOC did not overcome the inhibitory effect of either ZK734 or ZK299 [Fig. 2(B)].

The effect of P could also be demonstrated in tissue explant cultures. In a preliminary experiment it was found that treating ovariectomized rabbits with P for 4 days prior to explant culture of endometrial pieces neither increased the baseline level of 42 kDa synthesis nor the ability of the tissue to respond to P in culture; in fact, there was a decrease in the response to P in culture (data not shown). Since the effect in culture occurs within 24 h of hormonal stimulation and since there are profound changes in the cellular composition of the rabbit uterus following 4 days of progestin stimulation [13] it was decided that a 24 h *in vivo* stimulation might be more appropriate. Again, *in vivo* treatment with P failed to enhance the synthesis of 42 kDa protein in explant culture and the amount of stimulation induced by addition of P in culture was slightly decreased (Fig. 3).

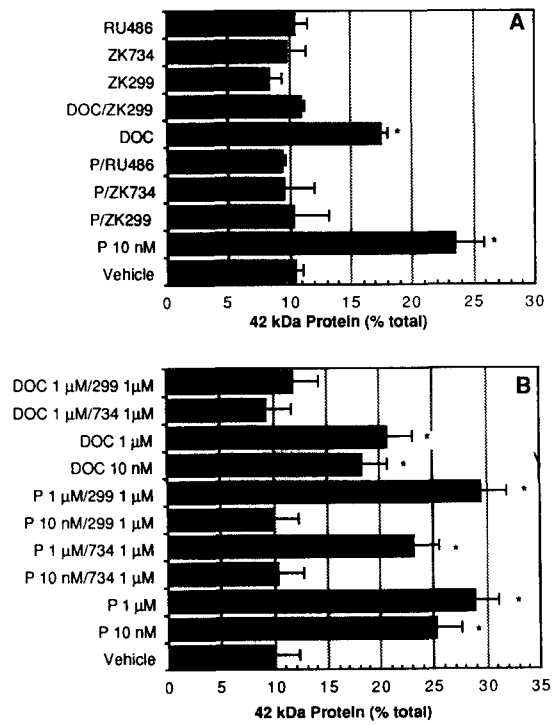

Fig. 2. Effects of antagonists on stimulation of 42 kDa protein synthesis. Cells were cultured for 3 days before addition of hormones and [^{35}S]methionine; media samples were collected 24 h later. (A) P or DOC were added at 10 nM. Antagonists, ZK299, ZK734 or RU486 were applied at 1 μM. (B) Steroids were added at the indicated concentrations to yield 100-fold concentration of antagonist or equimolar concentrations of hormone and antagonist. Mean ± SEM is presented; *treatment means that differed from the vehicle treatment mean, $P < 0.05$.

DISCUSSION

Cultured cells continued to synthesize 42 kDa protein during 96 h of continuous P treatment. It was anticipated that estrogen treatment

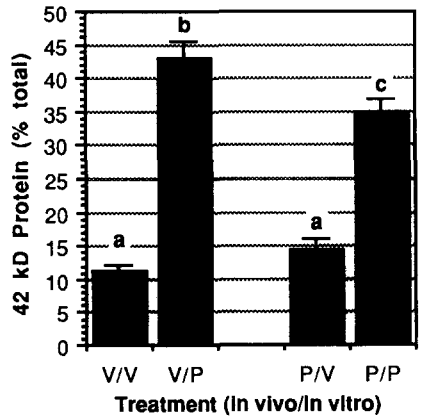

Fig. 3. Effect of *in vivo* P treatment. Rabbits were injected with 5 mg P; 20 h later their uteri were removed and placed in explant culture in the presence or absence of 10 nM P. The cultures were incubated for another 20 h in the presence of [^{35}S]methionine. Treatments: V/V, vehicle *in vivo* and *in vitro*; V/P, vehicle *in vivo* and P *in vitro*; P/V, P *in vivo* and vehicle *in vitro*; P/P, P *in vivo* and *in vitro*. Means ± SEM; those means with different letters are different from each other, $P < 0.05$.

would enhance the cellular P receptor content during this period of prolonged stimulation and thereby enhance the response to P, particularly during the later days of treatment. However, E_2 had no such effect. Nonetheless, there was substantial induction of the protein throughout the culture period that would allow for its accumulation, thereby facilitating its purification. The medium used in these studies contained the pH indicator, phenol red; since phenol red is known to act as an estrogen in cultures of breast cancer cells [14] it may be that P receptor content of the cells was already fully stimulated by that compound. When cells were grown in the absence of phenol red, E_2 enhanced both baseline levels and P-induced levels of 42 kDa protein to similar extents.

The steroid analogs RU486 and ZK299 are reported to have both antiP and antiglucocorticoid activities; they bind to both P and glucocorticoid receptors. ZK734 is reported to be more specific for the P receptor and therefore shows little or no antiglucocorticoid activity [15, 16]. All 3 antagonists completely blocked the effect of P when they were present at a 100-fold concentration. However, when the antagonist and P were in equimolar concentrations, the antagonistic effects were overcome. In contrast an equimolar concentration of DOC did not overcome the blocking effect of the antagonists. These results are consistent with the concept that the 42 kDa protein synthetic response is mediated by the P receptor and that the effect of DOC results from its ability to bind to the P receptor [17].

P treatment *in vivo* did not result in an increase in 42 kDa synthesis but rather, it decreased the responsiveness of the tissue to P in culture. These observations pose a paradox. The effect of P in culture has been observed during a 20–24 h treatment period while a similar treatment *in vivo* has no apparent effect. It may be that synthesis of the protein under investigation requires additional stimulation provided by the "shock" of tissue culture. This would be expected if 42 kDa protein represents a product of the decidualization reaction of the rabbit uterine stroma, a process that is initiated by injury to the uterine luminal epithelium during embryo implantation [18]. The widely observed spontaneous decidualization of rat endometrial cells in culture supports this notion [19–21]. Alternatively, the *in vivo* treatment was in the form of a bolus injection of the steroid which is likely to deliver P to the target cell in a short pulse while the culture experiments were performed under the more physiologic condition of a prolonged, continuous presence of the hormone. Results following *in vivo* treatment with a depot progestin preparation are required to address this question.

To summarize, the studies performed to date show that the bioassay being developed is specific for a P receptor mediated response and that it can be used to screen for P antagonists. Endometrial stromal cells from 1 rabbit are sufficient to perform 50–100 individual tests. It is likely that this bioassay system will prove useful in continued studies on the mechanism of P action and can substitute for animal studies in the screening of potentially useful antiprogestin analogs.

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