Hormonal Control of Uteroglobin Secretion in Rabbit Uterus

INHIBITION OF UTEROGLOBIN SYNTHESIS AND MESSENGER RIBONUCLEIC ACID ACCUMULATION BY OESTROGEN AND ANTI-OESTROGEN ADMINISTRATION

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Investigations were conducted to quantify activity of uteroglobin mRNA and secretion of uteroglobin in rabbit uterus after administration of progesterone and 5 α -dihydrotestosterone, either alone or concomitantly with oestradiol-17 β and tamoxifen, a non-steroidal anti-oestrogen. Poly(A)-containing mRNA was isolated from the uterine tissue by extraction with phenol/chloroform, precipitation with ethanol and chromatography on oligo(dT)-cellulose. Cell-free translation *in vitro* of the poly(A)-containing mRNA was carried out in a wheat-germ lysate, and the product isolated by specific immunoprecipitation with anti-uteroglobin antiserum purified by affinity chromatography. Radioimmunoassay was utilized to determine uteroglobin content in the uterine flushings and tissue preparations. When given for 5 days, both progesterone (1 mg/kg per day) and 5 α -dihydrotestosterone (25 mg/kg per day) elicited a marked induction of uteroglobin secretion, which was accompanied with accumulation of uteroglobin mRNA in the tissue. Concomitant administration of oestradiol-17 β (50 μ g/kg per day) or tamoxifen (12.5 mg/kg per day) significantly decreased both progesterone- and 5 α -dihydrotestosterone-induced uteroglobin secretion, with a parallel decrease in the uteroglobin-mRNA activity. The decline in the uteroglobin content of the uterine flushes brought about by oestradiol-17 β or tamoxifen administration was not due to inhibition of secretion of this protein by the endometrial cells, since a simultaneous decrease occurred in the tissue uteroglobin content. After a 5-day pretreatment with progesterone (1 mg/kg per day), administration of oestradiol-17 β (50 μ g/kg per day) during the ensuing 4 days greatly accelerated the decay of the uteroglobin content in the uterine fluid.

Uteroglobin (Beier, 1968) or blastokinin (Krishnan & Daniel, 1967) is a small secretory protein of rabbit uterus whose synthesis is induced by steroids with progestational activity (Beier, 1968; Urzua *et al.*, 1970; Arthur & Daniel, 1972; Mayol & Longenecker, 1974; Torkkeli *et al.*, 1977; Jänne *et al.*, 1978). In addition to progestins, natural and synthetic androgens were also shown to elicit induction of uteroglobin synthesis (Feigelson *et al.*, 1977; Jänne *et al.*, 1978). Progesterone alone is sufficient to bring about stimulation of uteroglobin secretion (Urzua *et al.*, 1970; Arthur & Daniel, 1972; Mayol & Longenecker, 1974; Torkkeli *et al.*, 1977; Jänne *et al.*, 1978), whereas oestrogens alone elicit a minimal enhancement in the secretion of uteroglobin by the endometrial cells (Mayol & Longenecker, 1974; Feigelson *et al.*, 1977; Torkkeli *et al.*, 1977). When administered along with progesterone, small amounts of oestrogens have been reported to potentiate the action of progesterone on uteroglobin secretion (Rahman *et al.*, 1975), whereas higher doses of oestrogens seem

to oppose the effect of progesterone by decreasing the relative uteroglobin content (Bullock & Willen, 1974; Barfield *et al.*, 1976; Bullock, 1977). During pregnancy of rabbits, highly elevated uteroglobin concentrations begin to fall after day 5, and the relative concentration of this protein in the uterine fluid declines to very low values by day 9, in spite of a continually rising serum progesterone content (Urzua *et al.*, 1970; Bullock & Connell, 1973; Beier, 1974). With a specific radioimmunological assay, Mayol & Longenecker (1974) were able to detect uteroglobin as late as on day 12 of pregnancy, although in much lower concentrations than on day 5 or 6. The decline in the uteroglobin content that occurs after day 5 has been postulated to derive from enhanced oestrogen production in rabbits at this stage of pregnancy.

Hormonal control of uteroglobin induction seems to take place through synthesis of a specific mRNA, since, in general, the steroids that enhance uteroglobin secretion also bring about accumulation of utero-

globin-mRNA activity (Beato & Nieto, 1976; Atger & Milgrom, 1977; Jänne *et al.*, 1978). As far as the action of oestrogens on the uteroglobin-mRNA activity is concerned, both increase (Beato & Nieto, 1976) and decrease (Atger & Milgrom, 1977) have been reported. The present work was undertaken to clarify the role of oestrogens in the induction of uteroglobin secretion and to find out the underlying mechanisms by which oestrogens interfere with the action of progestational steroids in the control of uteroglobin synthesis.

Experimental

Animals

Young New Zealand white rabbits (2–2.5 kg body wt.) were utilized in all experiments. Where indicated, the drugs were injected subcutaneously in ethanol/propane-1,2-diol (1:9, v/v; 1 ml) at two separate sites in the back of the animals. Doses of the compounds and duration of the individual experiments are given in the legends to Tables and Figures. The rabbits were killed 18–20 h after the last treatment dose by injecting air into the marginal ear vein.

Materials

L-[³⁵S]Methionine (1000 Ci/mmol) and Na¹²⁵I (11–17 Ci/mg) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Progesterone (4-pregnene-3,20-dione), 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one) and oestradiol [1,3,5(10)-oestratriene-3,17 β -diol] were supplied by Steraloids, Waltham, NH, U.S.A.; Sephadex G-25, Sephadex G-75 and CNBr-activated Sepharose 4B were from Pharmacia AB, Stockholm, Sweden; DEAE-cellulose (DE-52) and glass-fibre filter discs (GF/C) were from Whatman, Maidstone, Kent, U.K., and oligo(dT)-cellulose (type 2) was from Collaborative Research Inc., Waltham, MA, U.S.A. Sodium dodecyl sulphate, spermine and poly(ethylene glycol) (Carbowax 6000) were purchased from Fluka, Buchs, Switzerland; phosphocreatine, creatine kinase (from rabbit muscle), ATP and GTP were from Boehringer Mannheim G.m.b.H, Mannheim, Germany; non-labelled amino acids, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Tris base were from Sigma Chemical Co., St. Louis, MO, U.S.A.; ultra-pure urea and density-gradient-grade sucrose were from Schwarz/Mann, Orangeburg, NY, U.S.A., and dithiothreitol was from Calbiochem, San Diego, CA, U.S.A. Tamoxifen [1-(4- β -dimethylaminoethoxyphenyl)-*trans*-1,2-diphenylbut-1-ene] was kindly given by Dr. J. S. Patterson, ICI Ltd., Macclesfield, Cheshire, U.K., and high-purity wheat germ was a gift from Mr. J. R. Ryan, International Multifoods, Minne-

apolis, MN, U.S.A. Other chemicals were of the highest available purity grade and were supplied by Merck A.G., Darmstadt, West Germany.

Glass-distilled water was used in the preparation of solutions. All glassware and solutions for RNA isolation and cell-free translation experiments were autoclaved and the solutions further passed through MillexTM filters (pore size 0.22 μ m; Millipore S.A., Molsheim, France). Gloves were worn during the experiments to avoid contamination from skin ribonucleases.

Isolation of uteroglobin and production of antibodies against uteroglobin

Homogeneous uteroglobin was isolated from uterine flushings of progesterone-treated rabbits by successive chromatographies on Sephadex G-75 and DEAE-cellulose, as previously described (Torkkeli *et al.*, 1977; Fridlansky & Milgrom, 1976). Monospecific anti-uteroglobin antibodies were raised in male guinea pigs (Torkkeli *et al.*, 1977). Blood was removed by cardiac puncture and anti-uteroglobin serum stored at –20°C until diluted for the use in the uteroglobin radioimmunoassay, or purified by affinity chromatography for selective immunoprecipitation of uteroglobin synthesized *in vitro* (see below).

Radioimmunoassay of uteroglobin

The protocol previously described for uteroglobin radioimmunoassay was rigorously followed (Torkkeli *et al.*, 1977). This technique involved the use of [¹²⁵I]iodouteroglobin as the tracer, a working dilution of 1/25 000 of the anti-uteroglobin antiserum, precipitation with poly(ethylene glycol) (final concentration 10%) of the antibody–uteroglobin complexes, and counting of the radioactivity in the precipitate in a γ -counter (LKB Ultra-Gamma; LKB Produkter, Bromma, Sweden). The smallest detectable concentration of uteroglobin was 0.05 ng/tube, or with 0.05 ml sample volumes 1 ng/ml of tissue extract or uterine flushing.

Uteri were flushed by passing a single 10 ml portion of sterile, ice-cold 0.15 M-NaCl three times through each uterine horn. The flushes were centrifuged at 5000 g for 15 min at 2°C and the supernatants used, after appropriate dilutions, for uteroglobin radioimmunoassay. Uterine tissue was homogenized in a buffer (1 g of tissue/5 ml of buffer) containing 10 mM-sodium phosphate buffer, 0.15 M-NaCl and 3 mM-NaN₃ (pH 6.8) with an Ultra-Turrax homogenizer. The resulting homogenate was centrifuged at 105 000 g for 60 min at 0–2°C to yield a soluble cytosol fraction, which was diluted and measured for uteroglobin content by radioimmunoassay.

Isolation of uterine RNA

Whole uteri were homogenized with an Ultra-Turrax homogenizer in a buffer (1g of tissue/2ml of buffer) composed of 100mM-Tris/HCl (pH9.0), 5mM- Na_2EDTA and 100 μg of heparin/ml. Then 1 vol. of buffer-saturated phenol was added and the mixture rehomogenized (saturation buffer: 100mM-Tris/HCl, pH9.0, 5mM- Na_2EDTA , 100 μg of heparin/ml and 0.5% sodium dodecyl sulphate). After addition of 1 vol. of chloroform, the sample was vigorously shaken at room temperature (22°C) for 20min. After clarification by centrifugation at 15000g for 5min, the aqueous phase was removed and the organic phase re-extracted with 1 vol. of the saturation buffer. The pooled aqueous phases were made 2% with respect to NaCl and precipitated with 2.5 vol. of ethanol at -20°C. The precipitate was collected by centrifugation at 13000g for 30min at 2°C and the pelleted RNA dissolved in 10mM-Tris/HCl (pH7.5) containing 0.5M-NaCl before oligo(dT)-cellulose chromatography.

In some experiments, endometrial polyribosomes were prepared and polyribosomal RNA was extracted, essentially as described by Beato & Nieto (1976).

Oligo(dT)-cellulose chromatography was performed as described by Aviv & Leder (1972), except that the intermediate wash with 0.1M-NaCl was omitted. The poly(A)-rich RNA was eluted with 10mM-Tris/HCl (pH7.5) and precipitated with cold ethanol, as outlined above. The precipitate was collected by centrifugation at 13000g for 30min and dissolved in a small volume of water for use in cell-free translation studies.

Rabbit globin mRNA

Rabbits were given daily subcutaneous injections of 2.5% (w/v) phenylhydrazine chloride solution (pH7.0; 0.3ml/kg body wt.) for 5 days and blood was removed 2 days later by cardiac puncture under barbiturate anaesthesia into heparin-coated syringes. Reticulocytes were harvested by centrifugation at 3000g for 10min and polyribosomes isolated as described by Nienhuis *et al.* (1974). The poly(A)-rich RNA, essentially globin mRNA, was isolated as outlined for the uterine RNA.

Cell-free translation of isolated mRNA fractions

The 30000g supernatant (S-30) preparations were isolated from wheat-germ lysate as described by Roberts & Paterson (1973) and stored in small batches in liquid nitrogen. All the samples were assayed in duplicate. The reaction mixture (0.1ml) for translation *in vitro* contained wheat-germ S-30 fraction (0.02ml), RNA preparation [0.5–3 μg in 0.04ml, concentration measured by A_{260} (20 A_{260} units $\approx$ 1 mg/ml)] and 0.04ml of the reaction mixture, which resulted in the following final concentrations

of the components: 20mM-Hepes (pH7.6), 2mM-dithiothreitol, 1mM-ATP, 20 μM -GTP, 8mM-phosphocreatine, 40 μg of creatine kinase/ml, 75mM-KCl, 2.5mM-magnesium acetate, 60 μM -spermine, 1 μM -[^{35}S]methionine and 20 μM each additional non-labelled amino acid. The reaction was allowed to proceed for 2h at 25°C and terminated by adding 0.02ml of non-labelled methionine (final concentration 7mM) and immersing the tubes in ice/water. Thereafter, the samples were centrifuged at 10000g for 10min at 2°C and the supernatants used for further studies.

The amount of [^{35}S]methionine incorporated into total proteins was measured by precipitation with trichloroacetic acid (10%, w/v). The precipitates were collected on glass-fibre filter-paper discs, washed with ice-cold 5% (w/v) trichloroacetic acid, dried at 60°C and counted for radioactivity by using Insta-Gel (Packard Chemical Co., Downers Grove, IL, U.S.A.) as the scintillation fluid. The incorporation of radioactive methionine into uteroglobin was measured by an immunoprecipitation technique (see below).

Purification of anti-uteroglobin antibody

The antiserum was purified by affinity chromatography on a column of Sepharose 4B that contained covalently linked uteroglobin. The matrix was prepared as follows: 5g of CNBr-activated Sepharose 4B was washed with 1 litre of 1M-HCl, 150ml of water and 150ml of 0.1M-sodium borate buffer containing 0.075M-NaCl (pH8.5), collected on a glass-fibre filter and suspended in 20ml of 0.1M-sodium borate buffer. The washed CNBr-activated Sepharose was immediately added to 10ml of uteroglobin solution containing 60mg of homogeneous protein. The mixture was shaken for 24h at 4°C, then filtered and washed with 4 $\times$ 30ml of 0.1M-sodium borate buffer. After washes, the affinity resin was suspended in 50ml of 1M-glycine (adjusted to pH8.5 with the sodium borate buffer) and gently shaken at room temperature for 2h. The resin was finally washed with several volumes of 0.1M-sodium borate buffer containing 0.4M-NaCl followed by 10mM-sodium phosphate/0.15M-NaCl/3mM- NaN_3 (pH7.5) buffer, in which solution it was suspended and stored at 0°C. Monitoring loss of uteroglobin from the supernatant and wash solutions revealed that 99% of the protein was coupled to Sepharose 4B, yielding a uteroglobin content of 11.9mg/g of the resin.

In a typical antibody-purification procedure, 50ml of anti-uteroglobin antiserum was incubated with 2.5g of the affinity matrix for 48h at 4°C. The suspension was then poured into a column and washed with 10mM-sodium phosphate/0.15M-NaCl/3mM- NaN_3 (pH7.5) until the A_{280} reached 0.1 unit. Low-

affinity antibodies were eluted with 0.1M-sodium acetate buffer (pH4.5), and the high-affinity antibodies were eluted with 50mM-HCl. For an immediate elevation of the pH in the latter elution, the 3ml fractions were collected in tubes containing 0.5ml of 1.5M-potassium phosphate buffer (pH8.2). The purified antibody solution was adjusted to pH7.5, divided into small batches and stored at -20°C until dilution and use in the immunoprecipitation experiments.

Immunoprecipitation of uteroglobin

The double-antibody technique was used. The first antibody was that purified by affinity chromatography, and the second antibody was prepared by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (26g/100ml) of anti-(guinea-pig γ -globulin) raised in sheep. Immunoprecipitation was performed from 0.02–0.04ml portions of the supernatants derived from the translations *in vitro* after centrifugation at 10000g. These samples were incubated for 24h at room temperature with purified anti-uteroglobin antiserum (0.2ml) diluted with a solution containing 10mM-sodium phosphate, 0.15M-NaCl, 3% (w/v) sodium deoxycholate and 3% (v/v) Triton X-100 (pH7.6). After this, partially purified anti-(guinea-pig) antiserum (0.2ml) was added and the incubation continued for additional 24h at 4°C . The precipitated material was collected by centrifugation (10000g, 15 min at 0°C) through a 0.5ml cushion of 1M-sucrose containing 3% (w/v) sodium deoxycholate and 3% (v/v) Triton X-100, and washed three times by this centrifugation technique. The final pellet was suspended in water (0.5ml) and counted for radioactivity with 10ml of Insta-Gel as the scintillation fluid. When immunoprecipitated material was analysed by polyacrylamide-gel electrophoresis, the washed pellets were dissolved in 10mM-sodium phosphate buffer (pH6.8) containing 8M-urea and 1% (w/v) sodium dodecyl sulphate. Non-specific trapping of [^{35}S]methionine, or precipitation of non-uteroglobin peptides, was monitored by parallel incubations in three ways: (i) by replacing uterine mRNA fraction with rabbit reticulocyte mRNA, (ii) by using γ -globulin fraction from non-immunized guinea-pig serum as the first antibody, and (iii) by performing the translation *in vitro* without added exogenous mRNA. Correction for non-specific precipitation was conducted according to the results from these experiments.

Polyacrylamide-gel electrophoresis

Protein electrophoresis was performed on polyacrylamide slab gels in the presence of 8M-urea and 0.1% sodium dodecyl sulphate as described by Swank & Munkres (1971). The total acrylamide concentration (T) and the percentage of the cross-linking (C), as calculated by the method of Miller *et*

al. (1975), were 12.5 and 7.7% respectively. After electrophoresis, the gels were cut into 2.5 mm slices, digested with 30% (v/v) H_2O_2 (0.5ml/slice) for 6h at 70°C and counted for radioactivity with Insta-Gel as the scintillation fluid. Non-labelled uteroglobin was in most instances used as the internal marker protein and was localized by staining with Coomassie Brilliant Blue (Weber & Osborn, 1969). In some experiments [^{125}I]iodouteroglobin was also used as the marker protein, and it was localized by direct counting of the slices in a γ -counter.

Other techniques

Protein was measured by the Folin procedure (Lowry *et al.*, 1951), with bovine serum albumin as the standard. Progesterone was measured by radioimmunoassay with a kit kindly given by Nordiclab Oy, Oulu, Finland. Statistical analysis of the results was performed by the Student's *t* test (two-tailed, *t*-independent).

Results

Purification of anti-uteroglobin antiserum by affinity chromatography

In order to guarantee the specificity of the immunoprecipitation procedure for the cell-free translations, uteroglobin antiserum was purified by affinity chromatography on a Sepharose 4B column containing covalently linked homogeneous uteroglobin. Fig. 1 illustrates a typical elution profile of the antiserum from the affinity matrix; 0.1M-sodium acetate buffer (pH4.5) was able to release minor amounts of the protein from the resin, but a lower pH (50mM-HCl, pH approx. 1.5) was required for the elution of the specifically bound antibody. Starting from 50ml of antiserum, typically about 80–100mg of purified antibody was recovered. When the flow-through fractions were re-treated with the affinity matrix, no protein was bound to the resin, nor were the flow-through fractions capable of binding [^{125}I]iodouteroglobin (results not shown). The purified antibody avidly bound [^{125}I]iodouteroglobin, but for convenience it was only used in the immunoprecipitation experiments and not in the radioimmunoassay for uteroglobin, where diluted native antiserum was employed.

Optimization of the immunoprecipitation of uteroglobin formed in vitro

The immunoprecipitation technique was rigorously optimized with regard to the amounts of the first and second antibody used. Fig. 2(a) shows the results from studies where increasing quantities of the affinity-chromatographically purified anti-uteroglobin antibody was utilized to precipitate either [^{35}S]methionine incorporated into peptides by the

wheat-germ lysate, or [¹²⁵I]iodouteroglobin. Interestingly, the antibody-titration curves for the product labelled *in vitro* and for [¹²⁵I]iodouteroglobin had different slopes. This may derive from the fact that the product *in vitro* was not identical with the native uteroglobin (see below). A similar titration experiment for the quantity of the second antibody is summarized in Fig. 2(b). The amounts of antibodies required for maximal precipitation of [³⁵S]-methionine-containing peptides were: the first antibody, 60 µg, and the second antibody, 3.0 mg. Consequently, these amounts were used throughout.

An estimate for the non-specificity in the immunoprecipitation was obtained from studies where

uterine mRNA was replaced with reticulocyte mRNA. Under the above conditions, 7–9% of radioactive peptides formed during translation of reticulocyte mRNA *in vitro* were precipitated (Fig. 2 and Table 1). When the first antibody was

Fig. 1. Purification of anti-uteroglobin antiserum by affinity chromatography

A 50 ml batch of the antiserum raised in male guinea pigs was incubated for 48 h at 4°C with 2.5 g of Sepharose 4B–uteroglobin affinity matrix and then packed into a column. After elution of the unbound material, the column was washed with 10 mM-sodium phosphate/0.15 M-NaCl/3 mM-Na₂SO₄ buffer until A₂₈₀ reached 0.1 unit. Low-affinity antibodies were eluted with 0.1 M-sodium acetate, pH 4.5, and high-affinity antibodies were eluted with 50 mM-HCl; 3 ml fractions were collected. Fractions nos. 24–26 were pooled and used for immunoprecipitation experiments.

Fig. 2. Optimization of the amounts of the first and second antibody for immunoprecipitation

(a) The first antibody titration curves. Indicated amounts of the first antibody were used to precipitate [¹²⁵I]iodouteroglobin (■, 20000 c.p.m.) or products formed during translation *in vitro* of uterine mRNA (●) or reticulocyte (globin) mRNA (▲). The amount of the second antibody used in this experiment was 3 mg/ml. Precipitation percentage for the translations *in vitro* refers to the ratio of the immunoprecipitated to trichloroacetic acid-precipitated radioactivity, and for [¹²⁵I]iodouteroglobin it is the percentage of maximally bound radioactivity. (b) The second antibody titration curves. Experimental conditions were as in (a), except that the amount of the second antibody was varied, and the concentration of the first antibody was 60 µg/tube.

Table 1. Immunoprecipitation studies on the products formed in the translation *in vitro* of uterine or reticulocyte poly(A)-containing mRNA

Translation of the uterine mRNA (1 µg) and reticulocyte mRNA (2 µg) *in vitro* and precipitation of the products were conducted as described in the text. The values are means of triplicate experiments.

Sample	Uterine mRNA (c.p.m.)	Reticulocyte mRNA (c.p.m.)
Total trichloroacetic acid-precipitable radioactivity	12200	36900
Immunoprecipitation with uteroglobin antibodies purified by affinity column	7170	2710
Excess uteroglobin (200 µg) added before immunoprecipitation	2560	2020
Uteroglobin antibodies replaced with non-immunized guinea-pig γ-globulin fraction	1820	1790

replaced with non-immunized guinea-pig serum, similar results were obtained. Excess of non-labelled uteroglobin added to the samples before immunoprecipitation was able to decrease the radioactivity to the value of non-specific precipitation, when samples from uterine mRNA translation were investigated (Table 1). By contrast, non-labelled uteroglobin did not influence the amount of labelled material precipitated from the samples derived from the translation of reticulocyte mRNA *in vitro* (Table 1). Collectively, these data show that non-specific precipitation of [35 S]methionine has a minor contribution to the immunoprecipitation results. In spite of this, a constant monitoring of non-specific precipitation was conducted in all the subsequent experiments, and the final values were corrected accordingly.

Analysis of peptides formed in the cell-free translation experiments

Although the above data suggested that uteroglobin was specifically measured by the immunoprecipitation technique from the translation medium, an analysis of the immunoprecipitated peptides was also carried out. As seen in Fig. 3, the [35 S]-methionine-containing immunoprecipitated material formed a single peak of radioactivity when analysed on a polyacrylamide slab gel in the presence of 8M-urea and 0.1% sodium dodecyl sulphate (Swank & Munkres, 1971). The mobility of the radioactive peak was independent of the presence of mercaptoethanol during sample preparation, and in agreement with the results of others (Beato & Nieto, 1976; Atger & Milgrom, 1977; Bullock, 1977) the translation product was slightly larger than the subunit

Fig. 3. Analysis of the immunoprecipitated material by polyacrylamide-slab-gel electrophoresis in the presence of 8M-urea and 0.1% sodium dodecyl sulphate

The arrows indicate the mobilities of the uteroglobin subunit obtained by mercaptoethanol/sodium dodecyl sulphate treatment (+ME), and of uteroglobin treated with dodecyl sulphate only (-ME) on the same slab. The direction of electrophoresis was from left to right.

Table 2. Inhibition of progesterone- and 5 α -dihydrotestosterone-elicited uteroglobin induction by administration of oestradiol and tamoxifen

The animals were treated for 5 days with daily subcutaneous injections of the compounds dissolved in 1.0ml of ethanol/propane-1,2-diol solution. The last injection was given 20h before death. The daily doses of the compounds were as follows: progesterone 2mg; 5 α -dihydrotestosterone 50mg; oestradiol 100 μ g; tamoxifen 25mg. Uteroglobin was measured from the uterine flushings by radioimmunoassay (Torkkeli *et al.*, 1977). Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. ^a Significance of the difference compared with the group treated with progesterone alone; ^b significance of difference compared with the group treated with 5 α -dihydrotestosterone alone: * P < 0.05; ** P < 0.01; *** P < 0.001.

Treatment	Uteroglobin concentration	
	(μ g/mg of protein)	Total (μ g)
Vehicle	0.27 $\pm$ 0.07 (10)	0.41 $\pm$ 0.10 (10)
Progesterone	176.32 $\pm$ 25.53 (10)	1376.0 $\pm$ 293.62 (10)
Progesterone + oestradiol ^a	15.37 $\pm$ 9.91*** (6)	44.61 $\pm$ 30.1** (6)
Progesterone + tamoxifen ^a	66.87 $\pm$ 21.2** (9)	170.13 $\pm$ 42.2*** (9)
5 α -Dihydrotestosterone	64.50 $\pm$ 8.7 (8)	295.5 $\pm$ 47.8 (8)
5 α -Dihydrotestosterone + oestradiol ^b	18.30 $\pm$ 8.1** (4)	43.1 $\pm$ 18.7** (4)
5 α -Dihydrotestosterone + tamoxifen ^b	1.60 $\pm$ 0.3*** (6)	3.3 $\pm$ 0.4*** (6)

of uteroglobin, but smaller than the native protein (Fig. 3).

Taken together, immunoprecipitation data and electrophoretic analyses of the precipitated peptides provided strong evidence for the notion that our translation assay *in vitro* was able to measure specifically uteroglobin-mRNA activity.

Influence of oestradiol and tamoxifen administration on the induction of uteroglobin by progesterone and 5 α -dihydrotestosterone

Daily administration of 2 mg of progesterone (1 mg/kg) for 5 days led to a 3000-fold elevation in the total uteroglobin content in the uterine fluid, with a concomitant 500-fold increase in the relative amount of uteroglobin, when expressed as μ g of uteroglobin/mg of uterine-fluid protein (Table 2). The corresponding values after a 5-day treatment with daily 50 mg doses (25 mg/kg) of 5 α -dihydrotestosterone were as follows: a 700-fold increase in the total uteroglobin content and a 240-fold enrichment in the relative amount of uteroglobin (Table 2). The potency of 5 α -dihydrotestosterone to induce uteroglobin synthesis was thus approx. 1% of that of progesterone calculated on the basis of daily doses of steroids given. This value agrees well with our previous findings about the ability of testosterone to elevate uteroglobin concentration (Jänne *et al.*, 1978).

When oestradiol or tamoxifen was administered alone, the following mean values were obtained for the uterine-fluid uteroglobin: oestradiol, total uteroglobin, 7.5 μ g, and 3.0 μ g of uteroglobin/mg of protein; tamoxifen, total uteroglobin, 0.6 μ g, and 0.4 μ g of uteroglobin/mg of protein.

Concomitant administration of oestradiol or tamoxifen with progesterone and 5 α -dihydrotestosterone resulted in a significant decrease in the secretion of uteroglobin (Table 2). In none of the cases was uteroglobin induction completely abolished, although the fall in the uteroglobin content was usually very dramatic. Thus combination of oestradiol (100 μ g or 50 μ g/kg daily) with progesterone led to uteroglobin excretion that was about 3% of that elicited by progesterone alone. Tamoxifen inhibited the action of 5 α -dihydrotestosterone to the same extent. Oestradiol and tamoxifen seemed to interfere with the induction of uteroglobin by progesterone and 5 α -dihydrotestosterone in a differential manner: oestradiol was more potent in inhibiting progesterone action, whereas tamoxifen was a stronger inhibitor of 5 α -dihydrotestosterone action (Table 2).

The effects of oestradiol and tamoxifen on the uteroglobin content in the uterine fluid were not due to inhibition of secretion of this protein, since a parallel but less marked decline also occurred in the tissue content of uteroglobin [mean values:

Fig. 4. Influence of oestradiol administration on the decline of uteroglobin content in the uterine flushings. The animals were treated first with progesterone (2 mg) for 5 days and then left without any treatment for indicated times ($-E_2$, ●), or treated with daily 100 μ g doses of oestradiol ($+E_2$, ○). The first oestrogen injection was given along with the last dose of progesterone. Relative uteroglobin (a) and total uteroglobin (b) concentrations are shown. Each point is a mean of duplicate experiments.

controls, 0.025; progesterone, 2.78; progesterone+oestradiol, 0.19; progesterone+tamoxifen, 0.45; 5 α -dihydrotestosterone, 0.45; 5 α -dihydrotestosterone+oestradiol, 0.31; 5 α -dihydrotestosterone+tamoxifen, 0.06 (μ g of uteroglobin/mg of soluble uterine protein)].

As illustrated in Fig. 4, administration of oestradiol (100 μ g daily) after a 5-day pretreatment with progesterone (2mg daily) greatly enhanced the rate of uteroglobin decay over the ensuing 4 days, compared with animals given progesterone and left without any steroid administration for the following 4 days. Again, the action of oestradiol was not

explainable by inhibition of uteroglobin secretion from the endometrial cells, since a parallel decline in the tissue content of uteroglobin was found (results not shown). Oestradiol did not influence the decrease of serum progesterone concentrations, since there was no difference in the progesterone values between the animals treated with oestradiol and those left without treatment after progesterone administration (Table 3).

Action of oestradiol and tamoxifen on the accumulation of uteroglobin-mRNA activity elicited by progesterone and 5 α -dihydrotestosterone

Induction of uteroglobin secretion by progesterone and 5 α -dihydrotestosterone was accompanied by a significantly elevated uteroglobin-mRNA activity in the uterine tissue, as shown in Table 4 ($P < 0.001$ in each case, when compared with control values). In accordance with the inhibition of uteroglobin secretion, both oestradiol and tamoxifen significantly decreased progesterone-mediated accumulation of uteroglobin-mRNA activity (Table 4). The action of oestradiol again seemed to be more pronounced than that of tamoxifen. With 5 α -dihydrotestosterone, only the action of tamoxifen on uteroglobin-mRNA activity was studied. It appeared that tamoxifen decreased the accumulation of uteroglobin mRNA in a fashion similar to its influence on the secretion of this protein (Table 4).

Collectively, these data indicate that at least one of the reasons for diminished uteroglobin secretion brought about by oestradiol and tamoxifen is a lower uteroglobin-mRNA content within the uterine tissue.

Table 3. *Progesterone concentrations in rabbit serum after administration of progesterone*

The animals were treated for 5 days with 2mg daily doses of progesterone and then left without any treatment (no treatment after progesterone), or treated with daily 100 μ g doses of oestradiol (E_2 after progesterone) for the ensuing 4 days. Results are means $\pm$ S.E.M. for three animals in each group.

Animal group	Serum progesterone (nmol/l)
Untreated controls	12.71 $\pm$ 6.10
No treatment after progesterone	
1 day	20.35 $\pm$ 6.87
2 days	16.29 $\pm$ 1.00
4 days	13.15 $\pm$ 1.45
E_2 after progesterone	
1 day	18.02 $\pm$ 4.54
2 days	14.42 $\pm$ 1.88
4 days	13.82 $\pm$ 6.64

Table 4. *Influence of oestradiol and tamoxifen administration on the accumulation of uteroglobin mRNA induced by administration of progesterone and 5 α -dihydrotestosterone*

The animals were treated for 5 days with daily subcutaneous doses of the compounds, the doses being as follows: progesterone 2mg; 5 α -dihydrotestosterone 50mg; oestradiol 100 μ g; tamoxifen 25mg. Total uterine RNA was isolated and poly(A)-containing RNA fraction separated, as described in the text. After translation *in vitro* in a wheat-germ lysate, labelled uteroglobin was isolated by immunoprecipitation. In each case, 1 μ g of poly(A)-rich RNA was used in the cell-free translation. The values are expressed as the amount of labelled methionine incorporated into uteroglobin under these conditions. Results are means $\pm$ S.E.M. for the numbers of experiments in parentheses. ^a Significance of the difference compared with the group treated with progesterone alone; ^b significance of the difference compared with the group treated with 5 α -dihydrotestosterone alone: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Treatment	Uteroglobin-mRNA activity	
	(pmol/uterus)	(pmol/g of tissue)
Vehicle	1.38 $\pm$ 0.51 (11)	0.72 $\pm$ 0.18 (11)
Progesterone	61.18 $\pm$ 11.17 (8)	8.72 $\pm$ 1.04 (8)
Progesterone+ oestradiol ^a	3.83 $\pm$ 1.23** (4)	1.08 $\pm$ 0.33*** (4)
Progesterone+ tamoxifen ^a	11.14 $\pm$ 3.90*** (7)	4.05 $\pm$ 1.30** (7)
5 α -Dihydrotestosterone	14.17 $\pm$ 2.71 (9)	3.70 $\pm$ 0.90 (9)
5 α -Dihydrotestosterone+ tamoxifen ^b	4.65 $\pm$ 0.81* (5)	1.69 $\pm$ 0.29 (5)

Discussion

In the present work, we have set up techniques *in vitro* for a specific assay of uteroglobin-mRNA activity from rabbit uterus. Instead of first preparing endometrial polyribosomes (Beato & Nieto, 1976; Atger & Milgrom, 1977), direct extraction of RNA from the whole uterine tissue was considered to be the best alternative for our purposes, owing to difficulties encountered in fast and reproducible separation of endometrial and myometrial tissues from rabbit uterus. In particular, these problems occurred with uteri of animals not treated with progestational steroids. Great attention was paid to guarantee the specificity of the methods developed for assay of mRNA coding for uteroglobin, and all the available data indicate that an acceptable specificity was reached. Along with the mRNA measurement, we used a specific and sensitive radioimmunoassay for determination of tissue and uterine-fluid uteroglobin concentrations (Torkkeli *et al.*, 1977). The problems inherent in electrophoretic methods mainly used for uteroglobin assays (low sensitivity, semi-quantitative nature, and susceptibility to changes in relative proportions of other uterine-fluid proteins) could be avoided by using the radioimmunoassay method. By combining the above assay techniques, the present study provides the first direct evidence that oestradiol and tamoxifen are strong inhibitors of uteroglobin induction by progesterone and 5 α -dihydrotestosterone, and do not merely decrease the relative amount of uterine-fluid uteroglobin (Beier, 1968; Bullock & Willen, 1974; Barfield *et al.*, 1976; Bullock, 1977). The decline in the uteroglobin content brought about by oestradiol and tamoxifen was, at least in part, due to inhibition of uteroglobin mRNA accumulation. By contrast, oestradiol and tamoxifen did not elicit their action on the uterine-fluid uteroglobin content by inhibiting the release of this protein from the endometrial cells, since parallel changes took place in the tissue and uterine-fluid uteroglobin values.

The present data are in agreement with previous results about the elevation of uteroglobin-mRNA activity by progesterone (Beato & Nieto, 1976; Atger & Milgrom, 1977; Bullock, 1977), and about the induction of uteroglobin synthesis by androgenic steroids (Feigelson *et al.*, 1977; Jänne *et al.*, 1978; Torkkeli *et al.*, 1978). Moreover, our previous notion that androgens also bring about their action on uteroglobin synthesis through enhancing uteroglobin-mRNA activity (Jänne *et al.*, 1978) was confirmed by studies with 5 α -dihydrotestosterone. We have previously suggested that progestins and androgens mediate their influence on uteroglobin synthesis via a mutual mechanism involving the progesterone receptor (Jänne *et al.*, 1978), since the binding of androgens to progesterone receptor

correlates with their ability to induce uteroglobin, and the action of androgens on the synthesis of this protein is not inhibited by concomitant administration of anti-androgens devoid of progestational activity. The current findings seem to support this hypothesis, because oestradiol and tamoxifen inhibited the action of both progesterone and 5 α -dihydrotestosterone, although quantitatively in a somewhat differential fashion.

The mechanisms by which oestradiol and tamoxifen interfere with the action of progesterone (and 5 α -dihydrotestosterone) are not known at present. It is not known whether the action of tamoxifen was due to its anti-oestrogenic properties, or to its weak oestrogen-like characteristics such as induction of progesterone-receptor synthesis (Horwitz & McGuire 1977; Jordan & Prestwick, 1978; Isomaa *et al.*, 1979). It is noteworthy that these compounds elicited their action at the level of uteroglobin-mRNA activity. A similar phenomenon seems also to occur in the regulation of uteroglobin-mRNA activity during pregnancy of rabbits (Bullock, 1977). Although the most plausible explanation for these findings would be inhibition of uteroglobin-mRNA synthesis by oestrogens and anti-oestrogens, further studies are required to exclude other alternatives such as enhanced rate of mRNA degradation, e.g. owing to activation of uterine ribonucleases. Inhibition of progesterone-induced uteroglobin synthesis by oestrogen administration does not seem to be a unique phenomenon where oestrogen behaves like an anti-progesterone, since oestradiol injection has been shown to inhibit the decidual-cell response in ovariectomized-rat uterus (Mayers, 1970).

The decline in the uteroglobin concentration after a 5-day progesterone treatment was relatively fast, the control values being reached by 4 days after the last dose of progesterone. This agreed with our previous results on the kinetics of uteroglobin disappearance after a single dose of progesterone or medroxyprogesterone acetate: uteroglobin values reached a peak at 12–16h, and returned to the control values within 30h after injection (Torkkeli *et al.*, 1977). When oestrogen treatment commenced on day 5 of progesterone treatment, a greatly accelerated rate for uteroglobin disappearance was found. One possibility for this finding could be that oestrogen administration blocks the synthesis of uteroglobin mRNA and that the decline in the uteroglobin content during oestrogen treatment would then be a reflection of a relatively short biological half-life of this mRNA. Another possibility is that oestrogen administration activates processes involved in the degradation of either uteroglobin mRNA or the protein itself. Further studies are required to elucidate these alternatives.

It has been repeatedly shown that administration of progesterone will decrease the cytosol pro-

gesterone-receptor concentration in a variety of species (cf. Baulieu *et al.*, 1975), including the rabbit (Isomaa *et al.*, 1979), when given after an oestrogen-priming period, and that oestrogen administration will stimulate the synthesis of new progesterone receptors (cf. Baulieu *et al.*, 1975). Moreover, the doses of tamoxifen used in the present study elevated the cytosol progesterone-receptor content in a fashion similar to that by oestrogens (Isomaa *et al.*, 1979). In our recent studies, we have found that the doses of progesterone used in the present work (1 mg/kg per day) did not abolish the action of simultaneously administered oestradiol or tamoxifen on the cytosol progesterone-receptor content, but a receptor increase analogous with that elicited by oestrogens and anti-oestrogens alone was achieved with the above combined drug treatments (V. Isomaa, H. Isotalo, M. Orava & O. Jänne, unpublished work). These 'favourable' receptor changes are in marked contrast with the present data showing depression of progesterone action by oestradiol and tamoxifen. Taken together, our results indicate that the control of the anti-progestational action of oestrogens is located distal from the progesterone-receptor synthesis, and that the receptor changes in this experimental system are incompatible with the current concepts of receptor-mediated steroid-hormone action.

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