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Timing of Anti-uterotrophic Effects of a Single Injection of Progesterone in the Oestrogen-stimulated Uterus: Comparison of the Sheep and Mouse*

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Abstract

Progesterone (P) is anti-uterotrophic in the oestradiol (E_2)-stimulated uterus of the mouse but not the sheep. To further understand this species difference, the time course of some early effects of a single injection of P on the E_2-stimulated uterus has been examined. In the mouse, P caused a prompt (2–4 h) decline in uterine wet weight and *in vitro* rate of RNA synthesis, and a more gradual decrease (4–12 h) in RNA : DNA and protein : DNA ratios and *in vitro* rate of protein synthesis in whole uterus. In the sheep, P had no effect at any time on either whole uterus or caruncular endometrium. The results support the concepts that (1) anti-uterotrophic effects of P in E_2-stimulated mice are mediated by a direct P effect on nuclear E_2 receptor level, and (2) in the sheep P is not anti-uterotrophic because it does not regulate nuclear E_2 receptor level.

Introduction

Progesterone (P) is not anti-uterotrophic in the oestradiol (E_2)-treated ovariectomized ewe. At 12 or 24 h after a single or the last of several P treatments, P decreases neither uterine weight, nor RNA : DNA and protein : DNA ratios, nor rate of protein synthesis in samples of whole uterus and endometrium. This inactivity of P contrasts markedly with the substantial decline observed in all these uterine parameters in the E_2-stimulated mouse at 24 h after a single P treatment (Miller *et al.* 1979; Stone *et al.* 1979). In our studies in the ewe, P caused a decline in the level of uterine cytosol E_2 receptor, as well as a marked decline in the cytosol level of its own receptor.

A single injection of P given to the ovariectomized, E_2-stimulated rat or hamster causes a rapid (2–4 h) decline in nuclear E_2 receptor and only a slower (8–24 h) reduction in the level of cytosol E_2 receptor. The anti-uterotrophic action of P in the rat occurs at about the time of the P effect on nuclear E_2 receptor, and too rapidly to be directly related to or caused by the decline in cytosol E_2 receptor level (Evans *et al.* 1980; Okulicz *et al.* 1981).

Our previous studies of the E_2-stimulated mouse did not reveal the time course of early uterine responses to P. Further, it remained possible that P induced early but transient changes in uterine metabolism in the ewe, which were no longer evident at 12 and 24 h after P treatment. In this regard, P causes an early inhibition of some uterine transcriptional events in the E_2-stimulated rabbit (Kokko *et al.* 1977), yet

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by 24 h after P treatment there is no change in uterine weight and uterine RNA : DNA and protein : DNA ratios, and an increase in the rate of protein synthesis (Miller *et al.* 1979).

In order to further understand the possible relationships between P effects on uterine steroid receptors and on uterine metabolic activities, we have examined the early time course of several uterine events following a single injection of P. The results for P effects on nuclear and cytosol levels of E_2 and P receptors in the uteri of the sheep and mouse are presented in the accompanying paper (Stone *et al.* 1982). In this paper, procedures for the *in vitro* measurement of protein and RNA synthesis are described, and the early time course of P-induced changes in these parameters as well as in uterine weight and RNA : DNA and protein : DNA ratios are determined. Comparisons of the data obtained for uterine weight and metabolic activities with the corresponding data for uterine steroid hormone receptors (Stone *et al.* 1982) provide some explanation of why the biological effects of P in E_2-stimulated sheep and mouse uterus differ.

Materials and Methods

Animals and Schedule of Hormone Injections

Mature Merino ewes were purchased from commercial sources and mature QS strain mice were obtained from the University of Sydney Animal House. All mice were bilaterally ovariectomized 2 weeks before the commencement of experiments. At the time of ovariectomy, mice had body weights in the range 25–36 g. Sheep were bilaterally ovariectomized 4–8 weeks before experiments and received priming injections of 40 μg E_2 every 2 weeks, the last priming injection being given 12–14 days before day 1. Ovariectomy was performed under Nembutal anaesthesia via dorsolateral (mouse) or mid-ventral (sheep) laparotomy.

Table 1. Schedule of oestradiol and progesterone injections given to groups of ovariectomized animals

All injections were given between 0800 and 0900 h and all animals were killed on day 3 or day 4, at 24 h after the day-2 injection (group 1, controls) or at 2, 4, 6 (sheep only), 8, 12 (mice only) or 24 h after the day-3 hormone injection(s) (groups 2 and 3). The daily doses of oestradiol (E_2) were 0·1 and 40 μg and of progesterone (P) 1·0 and 12 mg for the mouse and sheep, respectively. Results are given in Fig. 2 and Tables 2 and 3

Day of experiment	Treatment schedule for animal groups		
	1	2	3
1	E_2	E_2	E_2
2	E_2	E_2	E_2
3	—	E_2	E_2+P

Experiments were carried out in duplicate (mouse) or triplicate (sheep). In each experiment, 88 mice or 11 sheep were allotted at random to 11 treatment groups containing eight mice or one sheep. The schedule of E_2 and P injections given and killing times in relation to hormone treatments are indicated in Table 1. E_2 and P were dissolved in peanut oil and administered by separate injections subcutaneously (mouse, 0·1 ml) or intramuscularly (sheep, 1·0 ml). Mice were killed by cervical dislocation and ewes by bleeding and section of the cervical spinal cord.

The genital tracts were dissected from animals promptly after killing and packed in crushed ice. Each uterus was carefully separated from adjacent tissues, blotted and weighed.

Rates of Synthesis of Protein and RNA, and Tissue Protein : DNA and RNA : DNA Ratios

Portions of whole uterus comprising both endometrium and myometrium were prepared for incubations as follows. The uterine horns of mice were slit lengthwise with fine scissors. The complete left and right horns from mice were pooled in pairs, so that duplicate incubation flasks contained two left horns or two right horns from the same two mice. In the case of the ewe, transverse sections (1–2 mm thick) of uterus were taken with scissors from a point on the right horn about 3–4 cm from the uterotubal junction. From each of these sections, one tissue slice about 0·5 mm thick was collected using a Stadie–Riggs microtome. Eight slices were prepared from each uterus and allotted at random to four groups of two slices each per incubation flask. Eight endometrial caruncles were also collected from the right horn of each sheep uterus. A single longitudinal endometrial slice about 0·5 mm thick was collected from the luminal surface of each caruncle, using a Stadie–Riggs microtome. All preparation of tissues was carried out at 0–5°C.

Tissue samples were incubated in a shaking water bath at 37°C in 5 ml Eagle's (Basal) medium (Commonwealth Serum Laboratories, Melbourne, Vic.) under an atmosphere of 95% O_2 : 5% CO_2. One half of the flasks prepared for each treatment group were pre-incubated for 30 min, then incubated for a further 120 min in the presence of 1·0 μCi L-[4,5-^{3}H]leucine (1 Ci/mmol, Radiochemical Centre, Amersham, U.K.) to determine rates of protein synthesis. The remaining flasks were preincubated for 30 min and then incubated for a further 60 min in the presence of 1·0 μCi [5-^{3}H]uridine (28 Ci/mmol, Radiochemical Centre, Amersham, U.K.) to determine rates of RNA synthesis. At the end of each incubation, the tissues were washed promptly in ice-cold 0·9% NaCl solution and stored at −18°C for subsequent determinations of RNA, DNA, protein and tritium content.

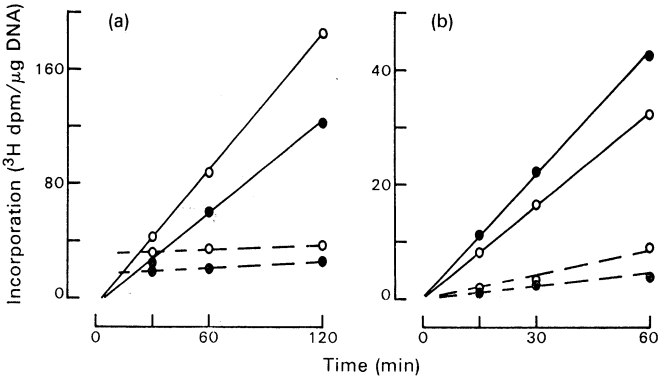

Fig. 1. Time course of *in vitro* incorporation of L-[4,5-^{3}H]leucine into protein and $HClO_4$-soluble fractions (*a*) and [5-^{3}H]uridine into RNA and 5′-nucleotide fractions (*b*) in mouse uterus. Mice received the treatments shown for groups 2 or 3 in Table 1 and were killed at 24 h after the day-3 hormone injection(s). Tissues were preincubated for 30 min, then incubated for 30, 60 or 120 min in the presence of L-[4,5-^{3}H]leucine (*a*) or 15, 30 or 60 min in the presence of [5-^{3}H]uridine (*b*). ○ Group 2. ● Group 3. Each point is the mean of four tissue pools. ——— Protein and RNA fractions. — — — $HClO_4$-soluble and 5′-nucleotide fractions.

Fractions containing RNA, DNA and protein were prepared and amounts of RNA, DNA and protein and of tritium incorporated into protein (incubations with L-[4,5-^{3}H]leucine) and RNA (incubations with [5-^{3}H]uridine) were determined as previously described (Miller 1979). Tissue RNA : DNA and protein : DNA ratios were determined for all tissue samples. Results are expressed as ^{3}H dpm (incorporated into protein or RNA) per microgram of DNA and mean tissue protein : DNA and RNA : DNA ratios.

Statistical Analysis of the Data

The significance of the effects of P was examined by Duncan's multiple range test (Steel and Torrie 1960) and is indicated in Fig. 2 and Tables 2 and 3.

Results

Initial experiments were carried out to validate these procedures for measuring *in vitro* rates of protein and RNA synthesis. In the case of protein synthesis, tissues were incubated for varying intervals in medium containing L-[4,5-^{3}H]leucine and the time course of tritium uptake into uterine protein and $HClO_4$-soluble fractions determined. Fig. 1*a* shows the results of such a trial with mice. The incorporation of tritium into protein was linear over 120 min, and during most of this interval the amount of tritium in the $HClO_4$-soluble fraction (and presumably of L-[4,5-^{3}H]leucine in the intracellular pool from which uterine protein is synthesized) remained almost constant. Further, different steroid treatments had little influence on the tritium concentration (dpm/mg protein or wet tissue) in this $HClO_4$-soluble fraction. (The

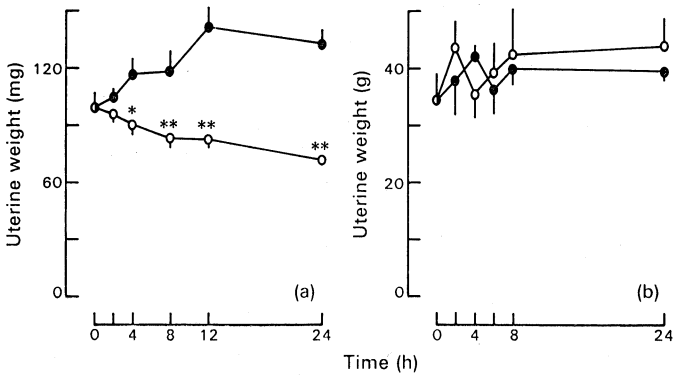

Fig. 2. Effects of a single injection of progesterone on uterine weight in the mouse (*a*) and the sheep (*b*). The design of the experiment is given in Table 1. Animals received oestradiol only (group 2, ●——●) or oestradiol + progesterone (group 3, ○——○) at 0 h on day 3. Control animals (group 1, 0 h) received no steroids on day 3. The results are means for eight mice or three sheep (uterine weights were recorded in only the second of duplicate mouse experiments). Vertical bars indicate 1 s.e. Standard errors not shown fall within the points as drawn: group 3 *v.* group 2: ** $P < 0{\cdot}01$; * $0{\cdot}01 < P < 0{\cdot}05$.

apparent treatment effect seen in this fraction in Fig. 1*a* results from steroid effects on tissue DNA concentration.) In the case of RNA synthesis, the incorporation of tritium into RNA was linear during the first 60 min of incubation (Fig. 1*b*). However, in this case the significance of the linear rate of ^{3}H incorporation into RNA is less clear, since the amount of tritium in the tissue $HClO_4$-soluble 5′-nucleotide fraction increased steadily during the incubation. The concentration of tritium in this fraction, obtained by chromatography of the $HClO_4$-soluble fraction on columns of Dowex 2X8-400 anion exchange resin, probably parallels the specific radiolabelling of the intracellular 5′-UTP pool from which RNA is synthesized (Miller and Baggett 1972*a*). Similar results were obtained with both L-[4,5-^{3}H]leucine and [5-^{3}H]uridine in corresponding experiments with sheep.

The E_2 given to mice on day 3 stimulated considerable uterine growth. Uterine weight and RNA : DNA and protein : DNA ratios all increased steadily to maximal

Table 2. Effects of a single injection of progesterone on protein : DNA and RNA : DNA ratios and *in vitro* rates of protein and RNA synthesis in whole uterus

The design of the experiment is given in Table 1. Results are means ± s.e., determined for mouse from eight tissue pools (protein : DNA and RNA : DNA ratios) and four tissue pools (protein and RNA synthesis), and for sheep from three animals. Group 1, control; group 2, oestradiol treatment only; group 3, oestradiol + progesterone treatment. Group 3 *v.* group 2: ** $P < 0{\cdot}01$; * $0{\cdot}01 < P < 0{\cdot}05$

Interval between day 3 treatment and killing (h)	Protein: DNA ratio	RNA : DNA ratio	Protein synthesis (^{3}H dpm/ μg DNA)	RNA synthesis (^{3}H dpm/ μg DNA)
		Mouse: Group 1		
0	7·3±0·3	0·78±0·02	156±15	33±3
		Group 2		
2	7·7±0·2	0·78±0·01	151±11	34±7
4	8·3±0·3	0·79±0·02	172±16	46±6
6	—	—	—	—
8	8·8±0·2	0·86±0·02	193±1	45±6
12	9·6±0·4	0·94±0·03	212±7	36±2
24	9·6±0·2	0·88±0·02	189±6	31±2
		Group 3		
2	7·5±0·3	0·77±0·02	148±10	27±3
4	7·6±0·2*	0·76±0·02	167±8	39±6
6	—	—	—	—
8	7·7±0·3*	0·74±0·02**	175±14	44±6
12	7·8±0·2**	0·76±0·02**	182±9*	44±3
24	6·6±0·2**	0·61±0·03**	104±5**	30±2
		Sheep: Group 1		
0	6·9±0·2	0·61±0·05	102±34	31±5
		Group 2		
2	6·6±0·8	0·57±0·04	85±21	33±11
4	6·9±1·0	0·65±0·04	92±18	35±10
6	7·0±0·2	0·66±0·02	103±32	32±9
8	7·9±0·6	0·71±0·09	96±37	28±11
12	—	—	—	—
24	7·6±0·6	0·67±0·04	75±24	32±9
		Group 3		
2	6·8±0·6	0·60±0·01	94±20	39±9
4	5·7±0·4	0·55±0·05	86±25	39±10
6	6·7±0·3	0·63±0·01	102±36	32±4
8	6·8±0·2	0·69±0·03	96±27	30±10
12	—	—	—	—
24	7·7±0·9	0·69±0·04	107±27	29±5

values at 12 h (increases over means at 0 h; uterine weight, 41%, $P < 0{\cdot}01$; RNA : DNA, 21%, $P < 0{\cdot}01$; protein : DNA, 31%, $P < 0{\cdot}01$), then remained

fairly constant between 12 and 24 h (Fig. 2 and Table 2). In contrast, the E_2 given on day 3 in the ewe appeared to cause only minor changes in uterine weight and RNA : DNA and protein : DNA ratios in whole uterus, and none of these apparent changes was statistically significant.

P abolished the growth response to day 3 E_2 in the mouse. In mice receiving P, uterine weight declined steadily between 0 and 24 h ($P < 0{\cdot}01$), whereas the RNA : DNA and protein : DNA ratios remained fairly constant between 0 and 12 h, then declined between 12 and 24 h ($P < 0{\cdot}01$). The significance of P effects at individual times is shown in Fig. 2 and Table 2. In comparison, giving P to ewes had no effect on uterine weight or RNA : DNA and protein : DNA ratios in whole uterus. In the mouse, P also reduced total uterine DNA by 14% at 24 h, but comparable data for the sheep were not obtained.

Table 3. Effects of a single injection of progesterone on protein : DNA and RNA : DNA ratios and *in vitro* rates of protein and RNA synthesis in sheep endometrium

The design of the experiment is given in Table 1. Results are means ± s.e. for three sheep. Group 1, control; group 2, oestradiol treatment only; group 3, oestradiol + progesterone treatment

Interval between day 3 treatment and killing (h)	Protein: DNA ratio	RNA : DNA ratio	Protein synthesis (^{3}H dpm/ μg DNA)	RNA synthesis (^{3}H dpm/ μg DNA)
		Group 1		
0	3·2±0·2	0·42±0·01	48±12	35±4
		Group 2		
2	2·7±0·2	0·37±0·01	42±11	31±8
4	3·1±0·4	0·41±0·03	51±14	35±9
6	3·7±0·4	0·48±0·01	66±17	41±1
8	3·1±0·4	0·45±0·05	50±17	31±9
24	3·4±0·4	0·41±0·04	44±12	35±6
		Group 3		
2	3·2±0·2	0·43±0·02	61±21	42±3
4	3·3±0·4	0·42±0·05	63±19	48±6
6	3·4±0·2	0·47±0·01	70±25	44±5
8	3·4±0·3	0·46±0·03	58±13	39±10
24	4·1±0·7	0·48±0·06	59±17	26±6

The *in vitro* rate of uterine protein synthesis in mice appeared to increase between 0 and 12 h regardless of whether P was given. However, the increase during this interval was significant only in mice that did not receive P, and in mice that did receive P the rate declined markedly between 12 and 24 h ($P < 0{\cdot}01$). Note that since P also decreased total uterine DNA between 12 and 24 h, the P effect on *in vitro* protein synthesis expressed per uterus was larger. In the ewe, E_2 given on day 3 did not stimulate the *in vitro* rate of protein synthesis, and P appeared to increase the rate at 24 h, but the variation between ewes within treatment groups for this parameter was large, for reasons unknown, and none of the effects of P on protein synthesis was significant. P effects on *in vitro* rate of RNA synthesis were generally

small. In the mouse, P appeared to depress RNA synthesis at 2–4 h, but not thereafter. Although the difference due to treatment was not significant at any one time (Table 2), there was, between 2 and 12 h, a highly significant interaction between P treatment and the linear effect of time ($P < 0{\cdot}01$; 1, 24 d.f.). In the ewe, P appeared to slightly stimulate RNA synthesis at 2–4 h, but none of the effects of E_2 or P was significant.

The effects of P on RNA : DNA and protein : DNA ratios and *in vitro* rates of protein and RNA synthesis in sheep endometrium are shown in Table 3. None of the effects of P was significant. There is no suggestion in the data that P inhibited any of these parameters, but rather that P may have further stimulated the sheep endometrium.

Discussion

The anti-uterotrophic action of P in the E_2-stimulated mouse uterus embraces a substantial decline in mean uterine cell RNA content (as reflected by changes in RNA : DNA ratio) and in the average amount of intracellular plus extracellular protein per uterine cell (as reflected by changes in protein : DNA ratio). Thus it seemed reasonable that measurements of RNA : DNA and protein : DNA ratios as well as *in vitro* rates of synthesis of RNA and protein in both whole uterus and endometrium in the ewe should reveal any significant anti-uterotrophic P effects during the first 24 h after P treatment. The changes observed in L-[4,5-^{3}H]leucine incorporation into protein probably were proportional to actual changes in uterine protein synthesis. However, the relationship between P-induced changes in [5-^{3}H]uridine incorporation into RNA and actual rates of RNA synthesis is less clear. The incorporation of [5-^{3}H]uridine into RNA is influenced by hormone effects on uridine transport into uterine cells and on salvage pathways for pyrimidine nucleotide synthesis, as well as by effects on RNA transcription. This problem of interpretation can be largely avoided by measuring the incorporation of tritium from [5-^{3}H]orotic acid or L-[methyl-^{3}H]methionine into RNA. However, orotic acid is incorporated at only a very low rate into all classes of uterine RNA, and L-[methyl-^{3}H]methionine labels only ribosomal and transfer ribonucleic acids. (For detailed discussions of the deficiencies of these and other methods for assaying RNA synthesis in the uterus, see Munns and Katzman 1971*a*, 1971*b*; Miller and Baggett 1972*a*, 1972*b*; Greenman 1977.) Alternatively, the level of transcription occurring in intact uterine cells can be followed indirectly by measuring *in vitro* RNA polymerase activities, chromatin template activity or both (Webster and Hamilton 1976; Kokko *et al.* 1977). Although the precise significance of the present data for RNA synthesis is unclear, the observed changes in [5-^{3}H]uridine incorporation into RNA almost certainly do reflect, at least in part, changes in actual rates of RNA synthesis.

Most of the measured effects of P in E_2-stimulated mouse uterus were evident by 4–8 h after P treatment; so that these metabolic responses followed closely behind P effects on the translocation of P receptor between cytosol and nuclear fractions and on nuclear E_2 receptor level (Stone *et al.* 1982). Conversely, at least some of the metabolic responses, as well as the effect on uterine weight, clearly preceded the P effect on cytosol E_2 receptor level, which became evident only at 8–24 h after P treatment (Stone *et al.* 1982). No cause-and-effect relationships between P effects on steroid receptors and uterine RNA and protein metabolism

are revealed in this study. However, the time course of these various P-induced responses in the mouse uterus do support the concepts that (1) the anti-uterotrophic action of P in the E_2-stimulated rodent uterus results from the direct actions of P and/or its receptor in the nucleus, including the prompt effect on nuclear E_2 receptor, and (2) the P effect on cytosol E_2 receptor replenishment is a consequence rather than a cause of the anti-uterotrophic actions (Koseki *et al.* 1977; Miller *et al.* 1979; Evans *et al.* 1980; Evans and Leavitt 1980; Okulicz *et al.* 1981). These presumptive conclusions are not inconsistent with the possibility that where E_2 and P both act on the rodent uterus for extended periods, the decline in cytosol E_2 receptor level induced by P may be a factor reducing uterine sensitivity to E_2 (Hsueh *et al.* 1976; Anderson *et al.* 1977).

P uniformly failed to inhibit any of the measured activities in the ewe in either whole uterus or endometrium at any time during the first 24 h after P treatment. Some of the results suggest a mild further stimulation of metabolic activity in the E_2-stimulated uterus. These results agree closely with our earlier findings (Miller *et al.* 1979; Stone *et al.* 1979), and show further that P fails to induce even early and transient inhibitory effects on uterine RNA and protein metabolism. In the E_2-stimulated rabbit, a single P treatment does cause some fluctuations in RNA polymerase I and II activities, including decreases in enzyme activities during the first 12 h after P treatment (Kokko *et al.* 1977). By 24 h after a single P treatment, P is not anti-uterotrophic in the rabbit, but rather causes some stimulation of protein synthesis (Miller *et al.* 1979). If P has similar early inhibitory effects on RNA polymerases in the ewe, they were not reflected in our measurements of RNA synthesis and mean cell content of RNA.

After P administration, there is a prompt redistribution of P receptor between cytosol and nuclear fractions of sheep endometrium (Stone *et al.* 1982). P, its receptor, or both, presumably act in the nucleus to initiate a spectrum of progestational responses, such as those required in the endometrium to support early development of the ovine embryo, and perhaps the induction of synthesis and secretion of specific uterine proteins associated with early pregnancy, analogous to the induction of uteroglobin in the rabbit (Isomaa *et al.* 1979; Bullock 1980; Isomaa 1981). However, unlike the case of the mouse and other laboratory rodents, P does not decrease nuclear E_2 receptor levels in E_2-stimulated ewes (Stone *et al.* 1982) and we suggest that this difference may explain why P is unable to inhibit the uterotrophic effects of E_2 in the ewe.

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