Effect of actinomycin D on uterine prostaglandin production and oestrous cycle length in guinea-pigs

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Summary. Intrauterine, but not systemic, administration of actinomycin D on Day 10 increased oestrous cycle length in guinea-pigs. Peripheral plasma progesterone levels remained elevated during these lengthened cycles presumably because luteal life-span had been extended. Prostaglandin (PG) F-2 α production *in vitro*, on Day 15, by the uterus of guinea-pigs which had received intrauterine actinomycin D was much lower than control values. This decrease in PG production was not due to lack of precursor, increased metabolism, re-direction of synthesis towards PGE-2, or a direct inhibition by actinomycin D of the conversion of arachidonic acid to PGs. The effects of actinomycin D treatment were not reversed by oestradiol.

It is proposed that actinomycin D prevents the increase in uterine PG synthetase levels that normally takes place after Day 11, thereby reducing uterine PGF-2 α synthesis and output *in vivo*, and resulting in luteal maintenance and longer oestrous cycles.

Introduction

There is much evidence that prostaglandin (PG) F-2 α is the uterine luteolytic hormone in several species of mammal (see Horton & Poyser, 1976). In the guinea-pig, utero-ovarian venous plasma levels of PGF-2 α increase from about Day 11 or 12 of the oestrous cycle (Blatchley, Donovan, Horton & Poyser, 1972; Earthy, Bishop & Flack, 1975). The *in-vitro* PGF-2 α production by homogenates of guinea-pig uterus utilizing endogenous precursor also increases from about Day 12, reaching peak production by Day 15 (Poyser, 1972). These *in-vitro* results have been confirmed by Wlodawer, Kindahl & Hamberg (1976) who used a microsomal preparation of guinea-pig uterus and exogenous arachidonic acid.

The difference in synthesizing capacity *in vitro* between days early and late in the cycle is not due to differences in endogenous substrate levels or differences in metabolism (Mitchell, Poyser & Wilson, 1977). It reflects a genuine increase in the amounts of PGF-2 α synthesized due to an increase in the amount (level) of prostaglandin synthetase within the uterus. The significance of this increase in enzyme levels was investigated by studying the effect of actinomycin D, a protein synthesis inhibitor, on the PG synthesizing capacity of the uterus and oestrous cycle length in the guinea-pig.

Materials and Methods

Virgin guinea-pigs weighing 500–800 g were examined daily and a vaginal smear was taken when the vaginal membrane was perforate. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification was at a maximum. All animals had exhibited at least 2 normal cycles before use.

Experiments

Experiment 1. On Day 10 of the cycle, 10 guinea-pigs were anaesthetized with pentobarbitone sodium (35 mg/kg). An incision was made in the abdomen and the junction of the two uterine horns was exposed. Each guinea-pig received a 1 ml injection of sterile, normal saline (9 g NaCl/l) into each uterine horn. For 5 of the animals the saline contained 20 µg actinomycin D. The body wall and skin of each guinea-pig were stitched, and the animals recovered in 6–8 h: none subsequently showed any side-effects from the operation or drug treatment. A further 5 guinea-pigs received a subcutaneous injection of 40 µg actinomycin D (Sigma, Poole) on Day 10 of the cycle: there were again no side-effects of treatment.

Oestrous cycle length was measured in all animals before and after treatment. Peripheral blood was withdrawn into a heparinized syringe by cardiac puncture on Mondays, Wednesdays and Fridays during the pre-treatment, treatment and post-treatment cycles. After centrifugation, the plasma was stored at -20°C until assay for progesterone.

Experiment 2. Fifteen animals were anaesthetized on Day 10 of the cycle, as in Experiment 1. Similarly, 10 guinea-pigs received an injection of 20 µg actinomycin D into each uterine horn, whilst the other 5 guinea-pigs (controls) received intrauterine saline only. A cardiac blood sample was taken from each animal on Day 15 of the cycle, for the analysis of plasma progesterone, and the guinea-pig was then killed. Prostaglandin production by the homogenized uterus and ovaries *in vitro* was measured, as described previously (Mitchell *et al.*, 1977) using 15 and 10 ml Krebs' solution respectively. However, arachidonic acid (final concentration 3 µg/ml) was added before incubation to the uterine and ovarian homogenates of 5 actinomycin D-treated guinea-pigs.

Experiment 3. Each of 10 guinea-pigs on Day 10 of the cycle received a subcutaneous injection of 0.4 ml saline ($N = 5$) or 40 µg actinomycin D in 0.4 ml saline ($N = 5$). A cardiac blood sample for progesterone determination was taken on Day 15. Prostaglandin production by the uterus and ovaries *in vitro* was determined as in Exp. 2 (without the addition of arachidonic acid).

Experiment 4. Ten guinea-pigs on Day 10 of the cycle were anaesthetized, and given an intrauterine injection of actinomycin D or saline as in Exp. 1. The 5 animals in each group were injected subcutaneously with 10 µg oestradiol benzoate (Sigma, Poole) from Days 11–14. On Day 15, a cardiac blood sample for progesterone determination was withdrawn before the animals were killed. Prostaglandin production by the homogenized uteri and ovaries *in vitro* (without the addition of arachidonic acid) was determined as before.

Experiment 5. Five guinea-pigs on Day 15 of the cycle were killed and their uteri removed. The two uterine horns were separated and weighed and each was homogenized in 7.5 ml Krebs' solution. Actinomycin D (20 µg) was added to the homogenate of one uterine horn from each pair. Prostaglandin production *in vitro* was measured as before.

Experiment 6. Six guinea-pigs on Day 10 of the cycle were anaesthetized, and each exposed uterine horn received an injection of actinomycin D (20 µg) or saline as in Exp. 1 (3 animals/group). The animals were killed on Day 15 of the cycle and the uteri and ovaries removed. The two uterine horns were separated, and the ovaries from each animal were also kept separate. Each uterine horn and ovary was weighed and homogenized in 10 and 5 ml Krebs' solution, respectively. To all homogenates was added 0.5 µCi [^3H]PGF-2α (sp. act. 160 Ci/mmol; Radiochemical Centre, Amersham, U.K.). Nicotinamide-adenine dinucleotide (NAD^+ ; 2 mM) and PGF-2α (5 µg/ml) were added to one uterine and one ovarian homogenate from each animal. All homogenates were incubated, and prostaglandins were extracted as in Exp. 2. The dried extracts were then each dissolved in 0.15 ml methanol and subjected to analysis by thin-layer chromatography by methods described previously (Maule Walker & Poyser, 1978). The percentage metabolism of PGF-2α was calculated from the radioactivity recovered, though a correction had to be made for the metabolite due to a loss of a tritium atom from the carbon at position 15.

A reference plate onto which had been spotted 10 µg PGF-2α and 10 µg 13,14-dihydro-15-oxo-PGF-2α was also analysed and the R_F values of PGF-2α and its main metabolite were calculated.

Assay procedures

Progesterone was measured by radioimmunoassay using an antibody raised and tested by Dighe & Hunter (1974) and assessed for its suitability for use in the laboratory by Poyser & Horton (1975). The antibody had adequate sensitivity (limit 20 pg) and specificity (see Table 1 in Poyser & Horton, 1975) and the results obtained in the present study were in the expected range. The intra- and inter-assay coefficients of variation were 10.8 and 12.5% respectively.

PGF-2α was measured by radioimmunoassay using an antibody developed in this laboratory (Dighe, Emslie, Henderson, Rutherford & Simon, 1975). This antibody does not distinguish between PGF-2α and PGF-1α. However, previous studies which have identified, by gas chromatography and mass spectrometry, the PGs formed by homogenates of guinea-pig uterus showed that neither PGF-1α nor PGE-1 are produced, since the guinea-pig uterus contains very little ω-dihomo-γ-linolenic acid (the precursor of the 1-series prostaglandins) compared with arachidonic acid (the precursor of the 2-series prostaglandins) (see Mitchell *et al.*, 1977). Consequently, in this study, it was highly probable that only PGF-2α was being measured. The control samples had values in the expected range and parallelism was observed for all samples assayed. The detection limit was 40 pg. The intra- and inter-assay coefficients of variation were 10.7 and 10.3% respectively.

PGE-2 was also measured by radioimmunoassay using an antibody, purchased from the Pasteur Institute (Paris), which has previously been tested and found suitable for use in this laboratory (Mitchell *et al.*, 1977). It cross-reacts significantly (15.1%) with PGE-1 but, for the reasons given above, it is highly probable that only PGE-2 was being assayed. Again, the results from the control samples were in the expected range and parallelism was shown by all samples in the assay. The detection limit was 4 pg. The intra- and inter-assay coefficients of variation were 10.3 and 13.5% respectively.

Statistical tests

Results, expressed as mean $\pm$ s.e.m., were analysed by Student's *t* test.

Results

Uteri removed from all animals were normal in gross appearance irrespective of whether they had received actinomycin D or not. Corpora lutea were adjudged to be functional if peripheral plasma progesterone levels were >1 ng/ml.

Experiment 1

As shown in Table 1, an intrauterine injection of actinomycin D on Day 10 significantly prolonged both luteal function ($P < 0.01$) and oestrous cycle length ($P < 0.01$), the increases being approximately 2-fold. Plasma progesterone levels, vaginal opening and vaginal cornification indicated normal post-treatment cycles in only 3 of the 5 treated animals, but the other 2 animals had a normal cycle after the post-treatment cycle.

Experiments 2, 3 and 4

The results in Table 2 show that the intrauterine administration of actinomycin D significantly reduced uterine PGF and PGE production *in vitro*, and that this was not due to lack

Table 1. Mean $\pm$ s.e.m. length (days) of the oestrous cycle and period when progesterone levels are >1 ng/ml plasma in guinea-pigs (5/group) treated with actinomycin D

Treatment	Oestrous cycle			Progesterone levels >1 ng/ml		
	Pre-treatment cycle	Treatment cycle	Post-treatment cycle	Pre-treatment cycle	Treatment cycle	Post-treatment cycle
Saline (intrauterine)	16.6 $\pm$ 0.4	18.2 $\pm$ 0.6	16.8 $\pm$ 0.2	10.4 $\pm$ 0.2	10.4 $\pm$ 0.2	10.6 $\pm$ 0.6
Actinomycin D (intrauterine)	16.6 $\pm$ 0.6	32.6 $\pm$ 5.4*	17.3 $\pm$ 1.2†	10.0 $\pm$ 0.6	20.4 $\pm$ 2.2*	10.3 $\pm$ 0.7†
Actinomycin D (subcutaneous)	17.2 $\pm$ 0.2	16.6 $\pm$ 0.4	16.6 $\pm$ 0.2	11.0 $\pm$ 0.5	10.4 $\pm$ 0.7	10.2 $\pm$ 0.7

* $P < 0.01$ compared with all other groups.

† Mean of 3 guinea-pigs. The other 2 guinea-pigs had anovular cycles of 9 and 13 days, respectively, and plasma progesterone did not exceed 0.7 ng/ml.

Table 2. Mean $\pm$ s.e.m. production of prostaglandin (PG) F and E *in vitro* by the uterus and ovaries, and peripheral plasma progesterone levels, in guinea-pigs (5/group) on Day 15 after intrauterine (Exps 2 and 4) or systemic (Exp. 3) administration of actinomycin D

Treatment	PG production (ng/100 mg tissue)				Progesterone (ng/ml)
	Uterus		Ovary		
	PGF	PGE	PGF	PGE	
<i>Experiment 2</i>					
Control	112.2 ± 12.4	7.5 ± 1.2	26.1 ± 2.6	5.2 ± 0.6	0.4 ± 0.1
Actinomycin D	23.6 ± 5.1†	3.7 ± 0.8*	30.0 ± 3.0	6.4 ± 0.6	3.8 ± 0.7†
Actinomycin D plus exogenous arachidonic acid	27.1 ± 4.0†	3.4 ± 0.4*	33.4 ± 7.2	7.0 ± 1.6	5.4 ± 0.6†
<i>Experiment 3</i>					
Control	139.9 ± 17.3	6.6 ± 0.9	30.9 ± 4.4	4.9 ± 0.5	0.6 ± 0.1
Actinomycin D	122.0 ± 8.4	5.9 ± 0.7	30.7 ± 1.3	4.7 ± 0.4	0.7 ± 0.2
<i>Experiment 4</i>					
Control	139.7 ± 22.7	12.9 ± 1.5	43.7 ± 13.3	7.5 ± 2.2	0.4 ± 0.1
Actinomycin D	35.3 ± 5.3†	5.8 ± 0.8†	30.4 ± 4.6	5.0 ± 0.3	6.6 ± 0.6†

Compared with respective control group, * $P < 0.05$, † $P < 0.01$.

of precursor (Exp. 2). Prostaglandin production by the ovaries was not affected. Plasma progesterone levels in guinea-pigs treated with intrauterine actinomycin D were higher than in control animals. When the actinomycin D was given systemically (Exp. 3) PG production and progesterone levels were unaltered.

Treatment with oestradiol benzoate (Exp. 4) did not prevent the decrease in uterine PG production and the elevation in plasma progesterone levels in guinea-pigs treated with intrauterine actinomycin D.

Experiment 5

The amounts of PGF produced by uterine horns in the presence and absence of actinomycin D were 78.8 $\pm$ 12.3 and 83.4 $\pm$ 12.0 ng/100 mg tissue respectively. Similarly, the amounts of PGE produced were 10.0 $\pm$ 2.4 and 9.4 $\pm$ 2.8 ng/100 mg tissue, respectively.

Experiment 6

The extracts from the uterine samples which contained NAD^+ and exogenous $\text{PGF-2}\alpha$ produced two radioactive zones on the t.l.c. plates. The zone containing the larger proportion of radioactivity coincided with authentic $\text{PGF-2}\alpha$ ($R_F = 0.34$). The other zone had the same R_F value (0.58) as 13,14-dihydro-15-oxo- $\text{PGF-2}\alpha$. Percentage metabolism by both groups was about 18% (Table 3). There was no detectable metabolism (<5%) by uterine samples incubated without NAD^+ and exogenous $\text{PGF-2}\alpha$, since only one radioactive zone (at $R_F = 0.34$) was located on the t.l.c. plates.

Total metabolism of $\text{PGF-2}\alpha$ by the ovary, in the presence of NAD^+ and exogenous $\text{PGF-2}\alpha$, was similar to that by the uterus (Table 3). The significant difference in the total amount of $\text{PGF-2}\alpha$ metabolized by the ovaries was removed by correction for weight of tissue. Metabolism of $\text{PGF-2}\alpha$ by ovarian tissue in the absence of NAD^+ was not detectable (<5%).

Table 3. Mean $\pm$ s.e.m. percentage metabolism of [^3H]prostaglandin F-2 α by uterine and ovarian homogenates in the presence of NAD^+ (2 mM) and exogenous $\text{PGF-2}\alpha$ (3 $\mu\text{g/ml}$) from guinea-pigs treated with intrauterine actinomycin D

Treatment	Uterus		Ovary	
	Total	Per 100 mg tissue	Total	Per 10 mg tissue
Saline (control)	17.2 $\pm$ 2.2	4.6 $\pm$ 0.9	12.4 $\pm$ 0.8	2.4 $\pm$ 0.4
Actinomycin D	18.8 $\pm$ 5.9	4.2 $\pm$ 1.5	19.1 $\pm$ 1.5*	3.8 $\pm$ 0.7

* $P < 0.05$ compared with control group.

Discussion

The $\text{PGF-2}\alpha$ synthesizing capacity of the guinea-pig uterus *in vitro* increases from about 40 ng/100 mg tissue during mid-cycle to about 120 ng/100 mg tissue by Day 15 (Poyser, 1972). This study has shown that treatment of guinea-pigs with actinomycin D injected directly into the uterus on Day 10 prevents this increase in uterine prostaglandin production. Systemic administration of actinomycin D was without effect. The decrease in uterine $\text{PGF-2}\alpha$ production observed after intrauterine treatment was not due to increased metabolism of the $\text{PGF-2}\alpha$ or re-direction of synthesis towards PGE-2 . The decrease in uterine $\text{PGF-2}\alpha$ synthesizing capacity *in vitro* was reflected *in vivo* by the peripheral plasma progesterone levels remaining high for a longer period than normal, presumably due to prolongation of luteal function, and by increased oestrous cycle length. It would appear, therefore, that an increase in uterine $\text{PGF-2}\alpha$ synthesizing capacity is important for the luteolytic action of the uterus to occur and, if prevented, luteal regression does not take place. This reduction in uterine $\text{PGF-2}\alpha$ synthesizing capacity, resulting in decreased uterine $\text{PGF-2}\alpha$ output and luteal maintenance, has previously been observed during early pregnancy in the guinea-pig (Maule Walker & Poyser, 1974, 1978; Blatchley, Maule Walker & Poyser, 1975). These effects have been attributed to the action of an antiluteolytic factor secreted by the conceptus. However, the antiluteolytic factor and actinomycin D cannot be acting in the same manner to prevent the increase in uterine $\text{PGF-2}\alpha$ synthesizing capacity, since the former can be overcome by oestradiol (Maule Walker, 1976), while the latter cannot (present study). Treatment of sheep with intrauterine actinomycin D also extends luteal function and oestrous cycle length, and this too is not surmountable by oestradiol treatment (French & Casida, 1973). Whether actinomycin D affects PG synthetase levels in the sheep uterus is not known.

In ovariectomized guinea-pigs, oestradiol treatment increases the PGF-2 α synthesizing capacity of the uterus (Naylor & Poyser, 1975). Since oestradiol output from the guinea-pig ovary increases from Day 11 up to the end of the cycle (Joshi, Watson & Labhsetwar, 1973), oestradiol is probably the physiological stimulus which causes the increase in uterine PGF-2 α synthesizing capacity. Actinomycin D acts to inhibit DNA-dependent RNA synthesis, so this metabolic step would appear to be essential in the mechanism by which oestradiol increases uterine PGF-2 α synthesizing capacity. The ultimate effect of actinomycin D treatment is to prevent fresh protein synthesis including, therefore, the synthesis of enzymes. The present *in-vitro* studies showed that intrauterine actinomycin D treatment cannot seriously be interfering with the availability of arachidonic acid *in vitro* since the addition of exogenous substrate did not overcome the reduction in uterine PGF-2 α production. It is proposed therefore that actinomycin D prevents the increase in PG synthetase levels in the guinea-pig uterus, which normally takes place after about Day 11 under the influence of oestradiol released from the ovary. Furthermore, *in-vivo* findings would suggest that this increase in enzyme levels (Pathway 2; see Text-fig. 1) is critically important for increased uterine PGF-2 α -synthesis and output to occur, and consequently for luteal regression to take place. Since the synthesis of PGF-2 α and PGE-2 is reduced by actinomycin D, the enzyme affected in the prostaglandin synthetase complex may be fatty acid cyclo-oxygenase, although further study is required.

Text-fig. 1. Proposed control mechanisms involved in the synthesis of prostaglandins by the guinea-pig uterus.

The levels of free arachidonic acid in tissues are believed to be too low for PG synthesis to occur and arachidonic acid has therefore to be released from some bound source. However, the absolute level of the free arachidonic acid in the guinea-pig uterus available for conversion to prostaglandin is not known. Consequently, whether activation of Pathway 1 (see Text-fig. 1) in the guinea-pig uterus is a prerequisite for prostaglandin synthesis needs to be investigated.

Intrauterine or systemic treatment of guinea-pigs with actinomycin D did not affect PG production by the ovary, the nearest organ to the uterus. The amounts of PGF-2 α produced were similar to those reported by Sharma, Wilson & Pugh (1976). (Day 15 in this study is equivalent to about Day -2 in their study.) However, PGE-2 production was about 5 times lower in the present study. The reasons for this discrepancy are not clear, although different antisera were used in the radioimmunoassays. Sharma *et al.* (1976) found prostaglandin production by the ovary to increase sharply after Day -2. It would be interesting to see if this is also affected by local actinomycin D administration. In rat isolated Graafian follicles, the LH-induced increase in prostaglandin levels is prevented by pre-treatment with actinomycin D (Zor, Strulovici, Nimrod

& Lindner, 1977). These authors proposed that an increase in prostaglandin synthetase enzymes is responsible for the increase in prostaglandin levels in rat follicles.

In conclusion, the intrauterine administration of actinomycin D to guinea-pigs prevented the increase in uterine prostaglandin synthesizing capacity which takes place after Day 11. It is proposed that the actinomycin D, by inhibiting DNA-dependent RNA synthesis, prevented the increase in the amount of one or more enzymes in the prostaglandin synthetase complex. However, due to the lack of specificity of actinomycin D on protein synthesis, other mechanisms of action (such as a reduction in uterine oestradiol receptors and/or enzymes regulating arachidonic acid uptake and release) which contributed to the effect observed cannot be ruled out.

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