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Estradiol-17β and Progesterone: Effects on Guanylate Cyclase Activity in the Myometrium of Macaques (40864)[1]

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Abstract. Guanylate cyclase activity was determined in various subcellular fractions of myometrium (1) from rhesus monkeys spayed for at least 6 months and treated either with estradiol-17β (E_2) for 14 days or with E_2 for 14 days and then for 5 to 14 additional days with E_2 plus progesterone (P) and (2) from cynomolgus monkeys during the follicular and luteal phases of natural menstrual cycles. Plasma levels of E_2 and P were similar in the spayed rhesus monkeys treated with hormones and the naturally cycling cynomolgus monkeys. In the 100,000*g* supernatant and particulate fractions of macaque myometrium before and after treatment with Triton X-100, the specific activities (per milligram nitrogen or DNA) of the guanylate cyclase were less in the luteal than in the follicular phases of both natural and induced menstrual cycles. The hormonal effects appear specific for myometrial smooth muscle since there was no difference in the guanylate cyclase activity of intestinal smooth muscle (taenia coli) in the follicular and luteal phases.

The effect of varying concentrations of Mn^{2+} on myometrium at different phases of an induced menstrual cycle was studied after homogenization with 1 m*M* EDTA. Guanylate cyclase activity in the 100,000*g* supernatant and particulate were higher in the "follicular" than in the "luteal" phase at all levels of Mn^{2+} studied. At the lower levels of Mn^{2+} the addition of 3 m*M* Ca^{2+} increased guanylate cyclase activity in the 100,000*g* supernatant fraction and decreased this activity in the 100,000*g* particulate fraction. The Ca^{2+}-induced increment in guanylate cyclase activity was greater in "luteal" than "follicular" myometrium, whereas the Ca^{2+}-induced decrement in the activity of the particulate fraction was greater in "follicular" than in "luteal" myometrium. It appears that cyclic nucleotide metabolism in nonhuman primate myometrium varies significantly during the menstrual cycle.

The effects of ovarian steroids on the intermediary metabolism of the uterus have recently been reviewed (1–4), however, the metabolism of cyclic nucleotides in smooth muscle in general, and myometrium in particular, has not been extensively studied. Uterine smooth muscle metabolism is of particular interest because of the periodic changes in the physiology and biochemistry of this tissue during female reproductive cycles. Much of the experimental work on the uterus has been done with rats, but it is difficult to separate the endometrium from the myometrium in this species, and these two tissues have usually been analyzed together. Endometrium and myometrium are, however, very different in structure and function and can be expected to react differently to metabolic stimuli (5, 6). The uteri of macaques (*Macaca mulatta* and *Macaca fascicularis*) can be satisfactorily separated into endometrium and myometrium, and the levels of estradiol-17β (E_2) and progesterone (P) during the menstrual cycle are well established in these species (7–9). Thus, the relationships between plasma steroid levels and myometrial enzyme activities can be precisely assessed in these nonhuman primates.

Cyclic GMP has been implicated as a

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regulatory component in the expression of a number of hormone actions, in mitogen action, and in a variety of altered physiological states (10). Goldberg and Haddox (11) have suggested that the evidence to date favors a role for cGMP as a modulatory effector molecule. For example, the level of cGMP might be important in regulating the level of cAMP in myometrium. We have reported elsewhere that increases in the concentrations of cGMP in the physiological range increased the hydrolysis of cAMP by myometrium from pregnant rhesus monkeys (12).

Several groups (13–15) have reported that estrogen increases the cGMP level in uteri from spayed rats. We had previously observed that the activities of cGMP phosphodiesterase (supernatant and particulate) in macaque myometrium were lower during the lutual phase of the menstrual cycle than during the follicular phase (16). Preliminary data also suggested that the basal guanylate cyclase activity (without Triton) was lower in the luteal phase. In the study being reported here, we measured the guanylate cyclase activites before and after Triton treatment in whole homogenates, and in 100,000*g* supernatant and 10,000*g*, and 100,000*g* particulate fractions of homogenates from myometrium of monkeys under the influence of either E_2 or E_2 plus P. We also compared the effects of Ca^{2+} and Mn^{2+} on guanylate cyclase activity under these different hormonal conditions. Myometrium was obtained: (a) during artificial "follicular" and "luteal" phases of menstrual cycles that had been induced by sequential treatment of spayed rhesus monkeys (*Macaca mulatta*) with E_2 and E_2 plus P and (b) during natural follicular and luteal phases of the menstrual cycles of cynomolgus monkeys (*Macaca fascicularis*).

Materials and methods. Preparation of monkeys. To induce an artificial menstrual cycle in bilaterally ovariectomized rhesus monkeys, we implanted a 3-cm Silastic capsule of E_2 in each monkey and 2 weeks later added a 4-cm capsule of P (16). Both capsules were implated subcutaneously in the midscapular region. Implants of this size administered in this sequence produce plasma hormone levels within the normal range (except for the midcycle E_2 surge) found in naturally cycling rhesus monkeys (17, 18). In this study, the plasma E_2 during the artificial follicular phase was 40 to 100 pg ml^{-1}, and plasma P was less than 0.2 ng ml^{-1}. During the artificial luteal phase, the E_2 averaged 50 pg ml^{-1}, and the P levels were over 2 ng ml^{-1} (19, 17). "Follicular"-phase myometrial samples were obtained 12 to 14 days after E_2 had been implanted. "Luteal" phase biopsies were obtained 5 to 14 days after P had been implanted.

We also obtained uteri, ovaries, oviducts, intestinal smooth muscle (taenia coli), and blood samples during the natural follicular and luteal phases from a group of intact, adult cynomolgus monkeys. Results on samples dissected at 4° and room temperature were similar. These samples were obtained at random during the menstrual cycle. Plasma E_2 and P levels were determined, and these levels were correlated with ovarian and endometrial structure and the amount of ciliation in the oviducts (20); the menstrual cycle stages were then determined retrospectively. Myometrial samples were obtained during either the middle to late follicular phase or the midluteal phase. The composite hormonal patterns during the menstrual cycle in cynomolgus and rhesus monkeys are similar (8, 9).

Collection of tissue. A biopsy of myometrium was obtained during laparotomy and freeze-clamped within 3 sec; then the uterus was removed, cleaned, and cut into quarters. The endometrium was carefully cut away from the myometrium, from which any residual endometrium was scraped. The myometrium was sliced into small pieces and freeze-clamped 5, 10, and 15 min after excision. For collection of taenia coli, a section of colon about 20 cm long was removed and the intestinal tract was repaired. The taenia coli were rapidly dissected from the intestine and freeze-clamped 5, 10, and 15 min after excision. The samples were wrapped in aluminum foil and stored at −200°. Frozen tissue was always used for analysis; no difference in guanylate cyclase activity has been observed between

fresh and frozen samples of myometrium. At liquid nitrogen temperature, enzyme activities did not change over a 12-month period of storage. The enzyme activity in the 100,000*g* supernatant increased 16 ± 2 SE% between 3 sec and 5 min after excision, and no significant change in the activity of either the supernatant or the particulate fraction was found between 5 and 15 min.

Preparation of cellular fractions. The frozen tissue was weighed and pulverized at liquid nitrogen temperature. The pulverized tissue (10% wt/vol) was homogenized in 0.25 *M* sucrose−10 m*M* Tris ([hydroxymethyl]amino methane) plus 1 m*M* dithiothreitol (DTT) at 4° in a Polytron ST-20 for 10 sec at three-fourths the maximum setting. Since in some tissues DTT reportedly stabilizes the enzyme by inhibiting autoactivation of guanylate cyclase, 1 m*M* DTT was included in the homogenizing medium. Its presence had no effect on myometrial guanylate cyclase activity; DeRubertis and Craven (21) have also reported that DTT has no effect on the activity of either soluble or particulate guanylate cyclase of rat renal cortex. The effects of various levels and ratios of Mn^{2+} and Ca^{2+} were studied after homogenization in the presence of 1 m*M* EDTA. The subcellular fractions were prepared by differential centrifugation of the homogenate as previously described (12). If the fractions were not immediately analyzed, aliquots were quick-frozen in liquid nitrogen and stored at −200°. Activity in these fractions did not change for at least a year. The nitrogen content of the individual fractions was determined on a Kjeldahl digest with a Technicon autoanalyzer.

Guanylate cyclase. The guanylate cyclase activity was determined by a modification of the method of Nesbitt *et al.* (22). The reaction mixture (0.1 ml) contained 50 m*M* Tris−HCl (pH 7.6), 5 m*M* $MnCl_2$, 1 m*M* cGMP, 1 m*M* [α-^{32}P]GTP (0.2 μCi, specific activity 18−33 Ci/mmole), 0.1% bovine serum albumin, 5 m*M* creatine phosphate, 5 units of creatine phosphokinase, 5 m*M* theophylline, 1 m*M* DTT, and 60 to 155 μg of protein (30 μl). To determine total solubilized activity, we preincubated fractions with 1% Triton X-100 for 1 hr before assay (4°). Assays were run for 10 min at 37° and were terminated by the addition of 0.5 ml of [^{3}H]cGMP, followed immediately by 0.05 ml of 50% trichloroacetic acid (TCA). Tritiated cGMP and TCA were added prior to the enzyme to establish blank values and these values were subtracted from each experimental value. The purity of the [^{3}H]cGMP was routinely checked every 2 to 3 months (23). The loss of product due to cGMP-phosphodiesterase activity was less than 2% and was ignored. Linearity of the assay with time and protein concentration was established. The assay tubes were centrifuged for 10 min at 1750*g*, and the supernatant was applied to Bio-Rad 50W-X4 columns (0.9 × 15 cm containing 1.5 ml of the resin in H_2O). Each column was rinsed with two 0.75-ml aliquots of H_2O and then eluted onto an alumina column (0.8 × 10 cm containing 0.5 g of neutral alumina) with two 0.9-ml aliquots of H_2O; this H_2O wash was discarded. The labeled cGMP was eluted from each alumina column with 4 ml of 0.6 *M* Tris−HCl (pH 7.6) and counted in a liquid scintillation counter. The amount of [^{32}P]-cGMP was calculated from the specific activity of the substrate [^{32}P]GTP and corrected for the recovery of added [^{3}H]cGMP (approximately 70 to 75%).

Hormone levels. Estradiol-17β and P levels were measured by radioimmunoassay (17).

Statistical analysis (*t* test). Differences in means were not considered significant unless the *P* value was <0.05 (24).

Results. A difference in the guanylate cyclase activity (in terms of milligram of nitrogen) of a specific subcellular fraction of the follicular compared to the luteal phase could be due to a change in the total enzyme activity in the myometrial homogenate or to a change in the distribution of the activity in the subcellular fractions. Therefore, before comparing enzyme activities in the follicular and luteal phases we determined the distribution of the total activities in the different fractions. However, there was no difference in the percentage of total

activity in the various fractions of the two series. In both phases of the menstrual cycle 4 to 8% of the total activity of the homogenate was found in the 10,000*g* pellet with or without Triton treatment, 77 and 6.5% in the 100,000*g* supernatant and pellet, respectively, without Triton and 27 and 29 to 30% with Triton (duplicate determinations on two monkeys). Since most of the guanylate cyclase activity was in the 100,000*g* supernatant and particulate fractions, we did not continue the analysis of the 10,000*g* pellet.

The specific activity of guanylate cyclase in the 100,000*g* supernatant and particulate fractions was greater in the follicular than in the luteal phase in both induced and natural menstrual cycles, and in the presence and absence of Triton (Table I). We have reported elsewhere that nitrogen concentrations in luteal and follicular myometrium were similar, as were the DNA levels (16). Therefore, the differences in specific activities of the myometrial guanylate cyclase in the follicular and luteal phases of the menstrual cycle were not due to a change in concentration of nitrogen or DNA. The guanylate cyclase activites in the 100,000*g* supernatant and particulate fractions of intestinal smooth muscle (taenia coli) during an induced menstrual cycle were also assayed, and there was no difference in the activity in the follicular and luteal phases (Table I). The basal values (not stimulated) for myometrial smooth muscle and taenia coli assayed in standard medium were similar; however, Triton treatment increased the myometrial particulate activity eight- to ninefold and the particulate activity from the taenia coli two- to threefold.

To determine if the difference in enzyme activity during the menstrual cycle was fortuitious and only seen under the exact conditions observed for the experiments in Table I, a series of experiments were done on fractions from myometrium homogenized in the presence of 1 m*M* EDTA. The effects of increasing concentrations of Mn^{2+} on enzyme activity with and without added Ca^{2+} were compared in the two series (Figs. 1a, b); without added Mn^{2+} there was no detectable activity. At all three levels of Mn^{2+} studied, the guanylate cyclase activity was lower ($P < 0.05$) in the induced "luteal" than in the induced "follicular" phase in both the supernatant and particulate fractions. The guanylate cyclase activity in the 100,000*g* supernatant was maximal at 5 m*M* Mn^{2+} (Fig. 1a) and in the particulate at 1 m*M* Mn^{2+} (Fig. 1b). The effects of Ca^{2+} were similar to those already described in the literature for other tissues, however, with or without added Ca^{2+} there was always a difference between the luteal and follicular series ($P < 0.05$). The Ca^{2+}-induced increment in guanylate cyclase activity of the supernatant was greater ($P < 0.025$) in "luteal" than in "follicular" myometrium, whereas the Ca^{2+}-induced decrement in guanylate cyclase activity of the particulate fraction was greater ($P < 0.01$) in "follicular" than in "luteal" myometrium.

Discussion. An enormous amount of research on cyclic nucleotide metabolism has accumulated over the last decade. However, less work has been done on cGMP than cAMP and the regulation of guanylate cyclase activity still remains a key process to be defined in studies on the role of cGMP in cell physiology (28). Graff *et al.* (10) have suggested that cGMP-linked signals may be received, processed, and integrated into biological regulatory processes differently than cAMP-linked signals, despite the fact that analogous components (cyclases, phosphodiesterases, and protein kinases) have been identified for both cyclic nucleotides.

Almost 10 years ago several workers (29, 30) suggested that cyclic nucleotides were involved in the expression of estrogen action in uterine tissues. More recently, E_2 administration has been reported to increase cGMP and decrease cAMP in whole uteri of spayed rats (11, 14, 15); diethylstilbesterol increased the cGMP in myometrial-enriched uterine tissue from spayed rats (13). Kuehl *et al.* (14) reported that cyclic nucleotide levels in rat uteri varied during the estrous cycle. These data represent values for whole uteri (endometrium plus myometrium) and are not comparable to our results. We have previously reported

TABLE I. SPECIFIC ACTIVITY OF GUANYLATE CYCLASE IN THE 100,000g SUPERNATANT AND PARTICULATE FRACTIONS OF MYOMETRIUM AND TAENIA COLI FROM MONKEYS DURING THE MENSTRUAL CYCLE[a]

	Myometrium (nmole cGMP produced/mg N · 10 min^{-1})							
	Before Triton				After Triton			
	Supernatant		Particulate		Supernatant		Particulate[b]	
Series	Induced[c]	Cycling[d]	Induced[c]	Cycling[d]	Induced[c]	Cycling[d]	Induced[c]	Cycling[d]
Follicular	12.8 ± 0.8 (11)	9.8 ± 1.2 (10)	—	8.6 ± 1.1 (8)	—	9.4 ± 1.1 (10)	68 ± 6.0 (10)	66 ± 8.0 (8)
Luteal	6.6 ± 0.7 (11)	6.8 ± 0.6 (10)	—	4.7 ± 0.8 (7)	—	6.7 ± 0.3 (10)	38 ± 3.8 (10)	43 ± 5.0 (7)
P-follicular vs luteal	<0.005	<0.05		<0.025		<0.025	<0.005	<0.05
				Taenia coli				
Follicular	14.7 ± 1.5 (8)	—	6.6 ± 0.8 (8)	—	10.9 ± 0.9 (8)	—	14.6 ± 1.2 (8)	—
Luteal	13.7 ± 1.4 (6)	—	5.3 ± 0.6 (6)	—	12.4 ± 0.9 (8)	—	14.9 ± 0.7 (8)	—
P-follicular vs luteal	>0.10		>0.10		>0.10		>0.10	

[a] Values are means ± SE. Values in parentheses are number of biopsies, at least five animals in each series, duplicate or triplicate determinations on each biopsy.

[b] P value for increase in activity after Triton X-100: <0.005 in both the induced and cycling series.

[c] Rhesus monkeys spayed for at least 6 months and then implanted with silastic capsules that produced physiological levels of plasma hormones, i.e., 40 to 100 pg/ml estradiol-17β and <0.2 ng/ml progesterone in the follicular (estradiol treated) series and 5 to 12 ng/ml progesterone and 40 to 100 pg/ml estradiol-17β in the luteal series, (estradiol plus progesterone treated).

[d] Cynomolgus monkeys, the uteri were obtained during normal menstrual cycles. The progesterone level was <0.2 ng/ml in the follicular series and 4 to 15 ng/ml in the luteal series.

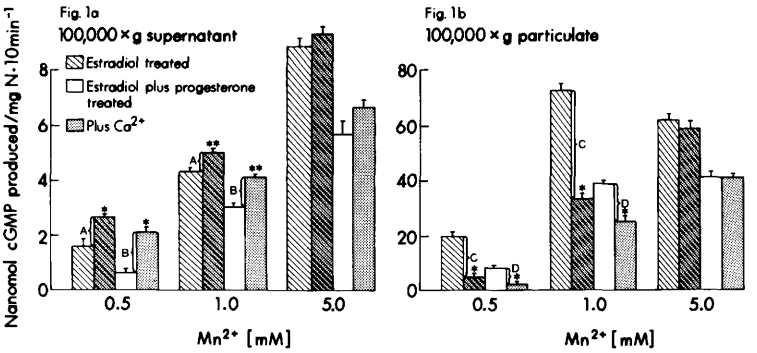

FIG. 1. Effect of Mn^{2+}, in the presence and absence of 3 m*M* Ca^{2+}, on the activity of guanylate cyclase in the 100,000*g* supernatant and particulate of rhesus myometrium during an induced menstrual cycle. Duplicate analysis on five monkeys; the parallel bars represent 1 SE. The muscle was homogenized in the presence of 1 m*M* EDTA and 30 μl of protein extract added to the assay medium. The particulate fraction was assayed after treatment with Triton. The *P* values for the differences between the follicular and luteal series were <0.05 in all instances. There was a difference in the effect of Ca^{2+} in the follicular and luteal phases at 0.5 and 1.0 m*M* Mn^{2+}, A < B ($P < 0.025$), C > D ($P < 0.01$). **P* value for addition of $Ca^{2+} < 0.01$ (statistical analyses on the basis of paired observations). ***P* value for addition of $Ca^{2+} < 0.05$ (statistical analyses on the basis of paired observations).

that in macaque myometria the cGMP concentration was greater during the induced "luteal" phase (5.9 ± 0.6 pmole/mg N) than during the induced "follicular" phase, (3.3 ± 0.4 pmole/mg N, $P = < 0.02$) (12). Our laboratory has also shown that in this tissue the specific activities of cGMP and cAMP phosphodiesterases (soluble fractions) were 20 to 40% lower in the luteal than in the follicular phases of both natural and induced menstrual cycles (16). The activities in the particulate fractions were the same in the follicular and luteal phases. The fact that in the luteal phase the cGMP levels were higher and both guanylate cyclase and cGMP phosphodiesterase activities were lower, suggests that during this phase the activity of guanylate cyclase *in vivo* decreased less than that of cGMP phosphodiesterase.

When the latent but expressible activity in the presence of detergents is considered, the total guanylate cyclase activity in the particulate fraction of most mammalian tissues, including macaque myometrium, is as large as or larger than that in the supernatant. The large amount of latent guanylate cyclase in myometrial homogenates suggests that the activation of this enzyme by some unidentified agent *in vivo* may regulate cGMP synthesis (25). However, in luteal myometrium we found that both the latent and the apparent guanylate cyclase activities were decreased. Both forms appear to be sensitive to the steroid hormones.

It is generally recognized that the soluble and particulate enzymes have biochemical properties that are different and appear to be regulated by different mechanisms (25, 26). However, both the soluble and particulate myometrial enzymes are sensitive to changes in plasma E_2 and P levels; i.e., differences between the luteal and follicular series were apparent in both fractions. Divergent effects of high levels of 1 to 5 m*M* Ca^{2+} on particulate and supernatant enzymes have been observed by others (26, 27, 31–33). These concentrations of Ca^{2+} are greater than the probable intracellular level of 3 to 10 μM.

Our data clearly show that the cyclic nucleotide metabolism of primate myometrium undergoes periodic changes during the menstrual cycle. Although there is no evidence to indicate whether the effect of progesterone is direct or indirect, our results strongly suggest that increases in plasma progesterone concentrations are responsible for the decrease in the activity of guanylate cyclase since enzyme activities were significantly lower in myometrium

during the luteal phase of both induced and natural menstrual cycles. This fact also suggests that the induced cycle is a satisfactory model for the study of the effects of sex steroids on myometrial metabolism. The progesterone effect appears to be specific for uterine smooth muscle because there was no difference in guanylate cyclase activity in intestinal smooth muscle (taenia coli) during the menstrual cycle.

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