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ESTROGEN-INDUCED ALTERATIONS IN CYCLIC NUCLEOTIDE AND PROSTAGLANDIN LEVELS IN TARGET TISSUE

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SUMMARY

Monitoring uterine cyclic GMP levels in the rat revealed that there was a statistically significant rise at proestrus from the values that are found at other stages of the cycle (estrus, metestrus, diestrus). Concurrent with the increase in cyclic GMP at proestrus was a decrease in cyclic AMP levels. Treatment of ovariectomized rats with estradiol benzoate (1 μg daily for 4 days) caused an increase in uterine cycle GMP levels from 0·04 to 0·10 pmol/mg tissue. Co-administration of progesterone (4 mg/day for 4 days) blocked this estrogen response.

Studies of prostaglandin levels in the cycling rat revealed a rise in PGF occurred at proestrus. That the alteration in PGF levels is estrogen related was evident by the fact that treatment of ovariectomized rats with estradiol benzoate (1 μg daily for 4 days) caused a large increase in PGF levels. This action of estrogen upon prostaglandin levels was blocked by co-administration of progesterone (4 mg/day for 4 days). There is clearly a temporal interrelationship between the action of estrogen to increase cyclic GMP and PGF levels. The ability of the prostaglandin synthetase inhibitor, indomethacin, to block the estrogen-induced increase in PGF, with no inhibitory action upon cyclic GMP formation, requires the formation of these two substances to be independent of each other, or the increment in PGF to follow that of cyclic GMP.

Since the classical work of Jensen[1] demonstrating the binding of estrogen to its cytosol receptor, there have been many reports about the nature of steroid receptor interactions. The report by Katzenellenbogen and Gorski[2] that estrogen is capable of causing the rapid synthesis of "induced protein" *in vitro* showed this to be one of the few estrogen-induced effects measurable *in vitro*. Although it is attractive to speculate that there may be a second messenger of estrogen action, similar to the role played by cyclic AMP for many pituitary hormones, no convincing evidence has been presented to show that this may be the case. Szego and Davis [3] have provided data permitting the suggestion that the action of estrogen may be associated with a rapid rise in uterine cyclic AMP levels. However, others [4], including ourselves [5], have been unable to confirm these findings. It has been suggested [6] that this rise in uterine cyclic AMP may be secondary to the action of catecholamines.

In considering the action of estrogens upon the uterus, certain effects, including increased glycogen deposition [7], cell proliferation [8] and release of lysosomal enzymes [7] are those that would be predicted to run counter to cyclic AMP action; *i.e.*, in the liver, cyclic AMP effects the conversion of phosphorylase b to the phosphorolase a, resulting in glycogenolysis [9], cyclic AMP inhibits cell proliferation [10] and the release of lysosomal enzymes in a number of tissues [11, 12]. On the other hand, these latter two actions, stimulation of cell proliferation [13] and release of lysosomal enzymes [11] have been shown to be induced by the nucleotide cyclic GMP. Thus, in retrospect, if estrogen action is associated with a cyclic nucleotide, cyclic GMP would be a more logical candidate than cyclic AMP to fit such a role.

For the above reasons, it would be most gratifying to state that our findings relating estrogen action to cyclic GMP were the result of such deductive reasoning. In actual fact, however, we must admit that the work reported here, demonstrating the ability of estrogen to stimulate uterine cyclic GMP, came about by chance, stemming from our interest in prostaglandins.

During the past few years our studies in the Merck Institute laboratories on the action of E-prostaglandins (PGEs) have led to the proposal that the PGEs act upon a specific membranous receptor [14] to regulate cell function by altering cyclic AMP levels [15]. On the other hand, the action of the F-prostaglandins, which exhibit a high degree of biological activity, in some instances opposite to those of PGEs, was not readily explained on the basis of an interaction with the PGE receptor to raise cyclic AMP. Upon learning of Dr. Nelson Goldberg's Yin-Yang concept [16], that in cells whose functions are bidirectional in nature these effects are expressed by the opposing actions of cyclic AMP and cyclic GMP, it became incumbent upon us to see whether the opposing action of PGEs and PGFs might not be expressed at the level of these nucleotides [17]. Dr. Goldberg's group kindly agreed to test this hypothesis, and found that treatment of the isolated estrogen-primed rat uterus with $PGF_{2\alpha}$ caused a rise in cyclic GMP levels within 45 s [17]. Meanwhile, we had set up the radioimmunochemical assay for cyclic GMP in our own laboratory, but were never able to convince

ourselves that the contractile effect of $PGF_{2\alpha}$ on the uterus *in vitro* was associated with a rapid rise in cyclic GMP levels. In defense of our concept, however, two laboratories have now shown that the contractile action of $PGF_{2\alpha}$ in the dog vein is associated with a rise in cyclic GMP. The opposite effect, the relaxing action of PGE_1, is associated with a rise in cyclic AMP levels [18, 19]. Concerning the uterus as it relates to cyclic GMP, we learned from Dr. L. Van Orden at the University of Iowa that estrogen is capable of raising PGF levels in this tissue [20], and began to wonder if the uterus of the estrogen-primed rat was the best choice for measuring the stimulation of cyclic GMP formation.

Accordingly, as shown in Fig. 1, we measured cyclic nucleotide levels in the uterus of the cycling rat. It is evident that cyclic GMP levels are essentially unaltered at metestrus, diestrus and estrus. However, a three fold increase was observed to occur at proestrus. In sharp contrast, levels of cyclic AMP are found to be depressed at proestrus, compared to other stages of the cycle. This latter finding is consistent with the report that adenylate cyclase activity reaches its minimum value at proestrus [21]. Since proestrus is the time when circulating levels of estrogen are at peak values, it was of obvious importance to see if the peak of cyclic GMP in the cycling rat occurred as a consequence of estrogen action. As demonstrated in Table 1, treatment of ovariectomized rats with estradiol-17β benzoate (1 μg s.c.) for four days prior to sacrifice resulted in an increase of uterine cyclic GMP levels from 0·044 to 0·108 pmol/mg of tissue. It is of interest to note that these two extremes correspond quite closely to the maximal and minimal values obtained in the cycling rat. Although administration of progesterone (4 mg s.c.) in the four day protocol at times gave an apparent increase in cyclic GMP levels, the results were highly variable and not statistically significant. On the other hand, coadministration of progesterone along with estrogen completely and consistently blunted the estrogen-induced increase in cyclic GMP levels. With these findings relating estrogen action to cyclic GMP, at that time considered preliminary in nature, the decision was made to continue and extend this work in collaboration with Dr. Goldberg's group. The results of this collaborative effort are the subject of several reports appearing recently in the literature [22–24].

Table 1. Effects of estradiol benzoate and/or progesterone on cyclic GMP levels in uteri of ovariectomized rats

Treatment	Weight of Uterine horn (mg)	Cyclic GMP pmoles/ Uterine horn	Cyclic GMP pmoles/ mg Tissue
Control (vehicle) s.c. 4 days	83 ± 4 (8)	3.72 ± .56 (8)	.044 ± .005 (8)
1 μg Estradiol Benzoate s.c. 4 days	193 ± 6 (4)	20.6 ± 3.1 (4)	.108 ± .018 (4)
4 mg Progesterone s.c. 4 days	111 ± 13 (4)	8.14 ± 2.45 (4)	.072 ± .018 (4)
1 μg Estradiol Benzoate + 4 mg Progesterone s.c. 4 days	128 ± 11 (4)	4.67 ± .22 (4)	.037 ± .004 (4)

Significance of differences among values in column at extreme right:
Estradiol vs. Control $p < .01$;
Estradiol vs. Estradiol + Progesterone $p < .01$.
All other differences are not significant. Mean values ± SE with number of observations in parenthesis.

A time course of the action of diethystilbestrol (DES) (100 μg, in oil, s.c.) in ovariectomized rats is shown in Fig. 2, wherein a statistically significant increase in cyclic GMP is noted within three hours, leading to a maximum 8-fold increase at 24 h. The decay of the estrogen-induced rise is represented in Fig. 3 where values are seen to return to control levels at 72 h. Returning to Fig. 2, a small but statistically significant decrease in cyclic AMP levels occurs as a consequence of DES action, when cyclic GMP levels are maximal. With more physiological doses of DES (1 μg s.c.) the same picture was obtained as with the higher dosage protocol.

By analogy to the action of agents that raise tissue levels of cyclic AMP, it would seem reasonable to predict that estrogen may elicit its action to raise cyclic GMP levels by activating guanylate cyclase.

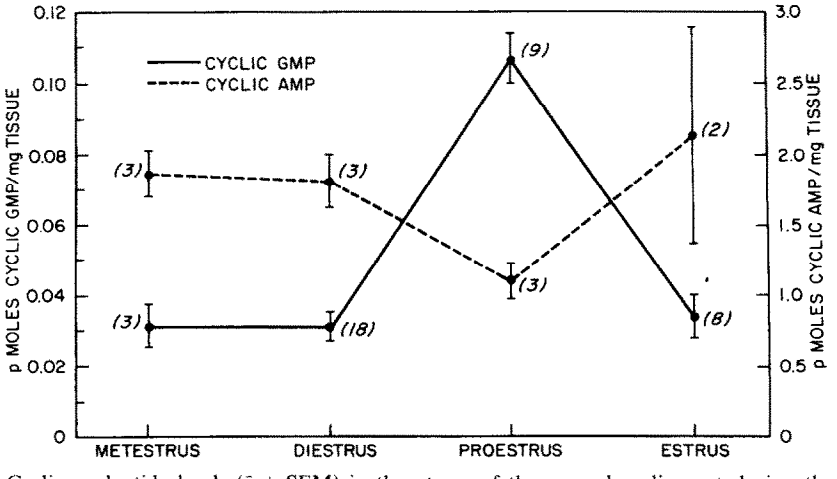

Fig. 1. Cyclic nucleotide levels ($\bar{x} \pm$ SEM) in the uterus of the normal cycling rat during the estrus cycle. Cyclic nucleotides were measured by *Method* 2 as described by Kuehl *et al.* [22].

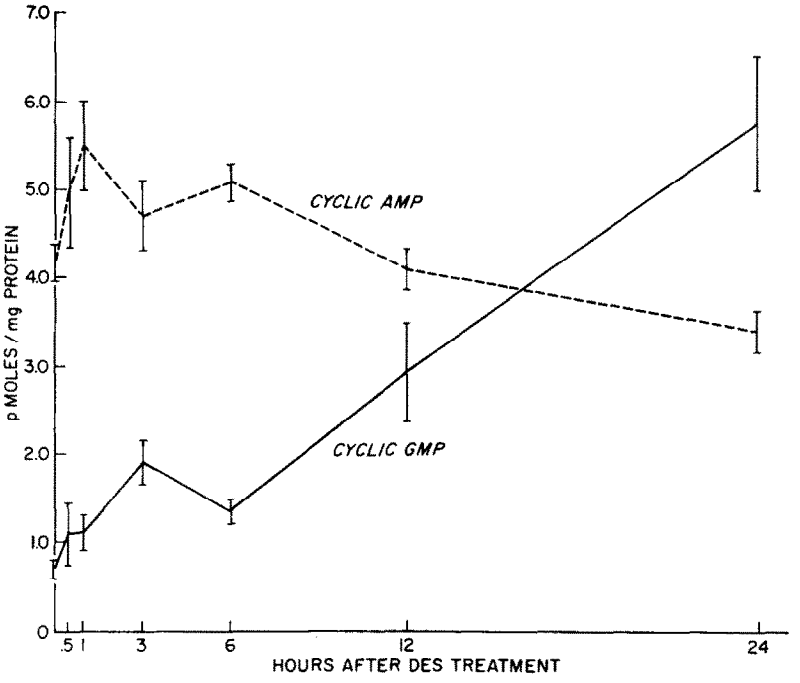

Fig. 2. Ovariectomized rats were treated with diethylstibestrol (100 μg, in oil, s.c.). Animals were killed at the times indicated and cyclic nucleotides were determined by *Method* 1 as described by Kuehl *et al.* [22].

However, uterine homogenates of estrogen-treated rats showed no greater ability to effect the conversion of GTP to cyclic GMP than those of ovariectomized controls. Although this study does not eliminate a stimulatory action of estrogen on cyclic GMP synthesis in intact cells, no induction of guanylate cyclase specific activity seems likely. One change that was uncovered that may account for the rise in uterine cyclic GMP levels was an apparent decrease (40–50%) in the rate of hydrolysis of cyclic GMP by phosphodiesterase in uterine homogenates of estrogenized animals [25]. This concept is more convincing since the decrease in the ability to degrade cyclic GMP was found to parallel the time course of the increments of the cyclic nucleotides observed after DES treatment.

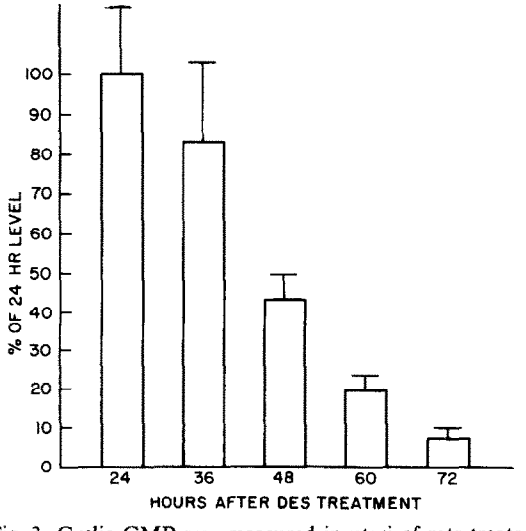

Fig. 3. Cyclic GMP was measured in uteri of rats treated as described in Fig. 2 at the times indicated [22].

If one evokes an intermediate role for cyclic GMP in estrogen actions it is obvious that this event must closely follow the interaction of estrogen with its receptor, and as with the formation of induced protein, this effect should be demonstrable *in vitro*. Using estrogen-sensitive pituitary GH_3 cells obtained from Dr. C. Sonnenschein, estradiol (10^{-8}M) was found to be capable of causing a rise in cyclic GMP from non-detectable levels to 0·7–1·3 pmol of cyclic GMP/10^8 cells within 15 min (Table 2). Similar *in vitro* studies with uteri of ovariectomized rats revealed that the same concentration of estradiol increased cyclic GMP 100–300% within 15 min. an effect blocked by the addition of progesterone (10^{-7}M)[24].

It is evident from the foregoing that the action of estrogen on the uterus is associated with an increase in cyclic GMP levels. Although it is attractive to speculate that this nucleotide is an intermediate in estrogen action, until it can be demonstrated that added cyclic GMP or an appropriate derivative can mimic estrogen action on uterine tissue, such a critical assignment cannot be made with any degree of confidence. However, the actions of cyclic GMP to stimulate cell proliferation and lysosomal release, as noted before, two established effects of estrogen on the uterus, are consistent with such a role.

Turning now to the prostaglandin aspect of estrogen action, we should now like to describe our

Table 2. Estrogen-sensitive GH_3 rat pituitary cells obtained from Dr. C. Sonnenschein, Tufts University School of Medicine, were grown in culture. 10^8 cells were incubated ± estradiol-17β (10^{-8}M), and cyclic nucleotides were measured by *Method* 2 [22]. In all but the first experiment cells were washed with incubation medium. In the last experiment cells were grown in the presence of 10^{-8}M estradiol-17β

Effects of Estradiol on Cultured GH_3 Pituitary Cells

	Estradiol	pmoles cGMP/10^8 cells	pmoles cAMP/10^8 cells
A. 15 minutes incubation ∓ 10^{-8}M estradiol			
Exp. I (unwashed cells)	–	3.1	93
	+	4.6	67
Exp. II	–	0.00	70
	+	0.75	100
Exp. III	–	0.00	204
	+	1.27	216
Exp. IV	–	0.01	161
	+	0.71	202
B. Cells cultured 7 days ∓ 10^{-8}M estradiol			
Exp. V	–	.29	242
	+	2.65	212

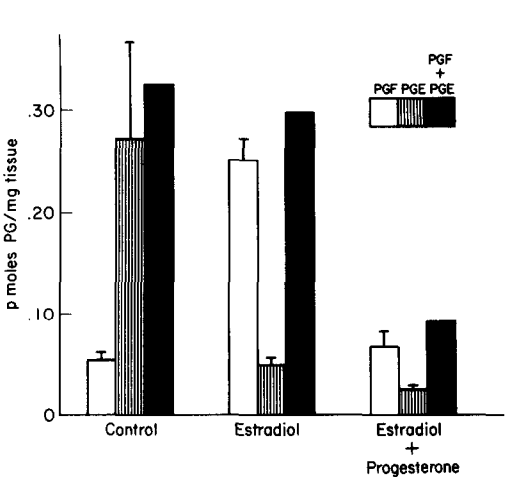

Fig. 5. Ovariectomized rats were given estradiol-17β benzoate (1 μg, in oil, s.c.) for four consecutive days. Uteri were removed 3–4 h after the final injection. Progesterone (4 mg, in oil, s.c.) was coadministered where indicated. Prostaglandins were measured as noted in Fig. 4.

more recent studies in the Merck laboratories relating prostaglandin production in the uterus to cyclic nucleotide levels, as a consequence of estrogen action. As shown in Fig. 4, measurement of PGEs and PGFs by radioimmunochemical techniques reveals that an increase in PGF is apparent at proestrus and generally persists throughout estrus. The direct relationship to alteration of cyclic GMP levels is also apparent from these data. There is a less dramatic but consistent increase in PGEs at proestrus.

Utilizing a protocol for administration of steroids to ovariectomized rats identical to that employed when cyclic nucleotides were measured (*i.e.*, 1 μg estradiol-17β benzoate, s.c. for four consecutive days ± 4 mg of progesterone) in three of five individual experiments estrogen administration was associated with an acute rise in PGFs, as typified by Fig. 5. In all instances, however, PGE levels were depressed as a consequence of estrogen action. Consistent with the inhibitory action of progesterone on estrogen-induced increases in cyclic GMP levels, progesterone is also seen to inhibit the stimulatory action of estrogen on PGF levels.

One conclusion that can be drawn from these studies is that there is a parallelism between the alteration of cyclic GMP and PGF levels as a consequence of estrogen action on the uterus. Recalling that $PGF_{2\alpha}$ is capable of inducing cyclic GMP formation in the dog vein, it is attractive to speculate that cyclic GMP may be secondary to PGF formation in the uterus. However, as shown in Fig. 6, treatment of estrogenized, ovariectomized rats with the prostaglandin synthetase inhibitor, indomethacin, completely blocked estrogen-induced prostaglandin production. In contrast (Fig. 6), no depression of cyclic GMP could be observed as a consequence of the action of indomethacin. The small synergistic effect apparent by the action of this prostaglandin synthetase inhibitor may be attributable to its theophylline-like ability to block phosphodiesterase activity.

We believe that it is possible to conclude from these studies that the effects of estrogen to stimulate

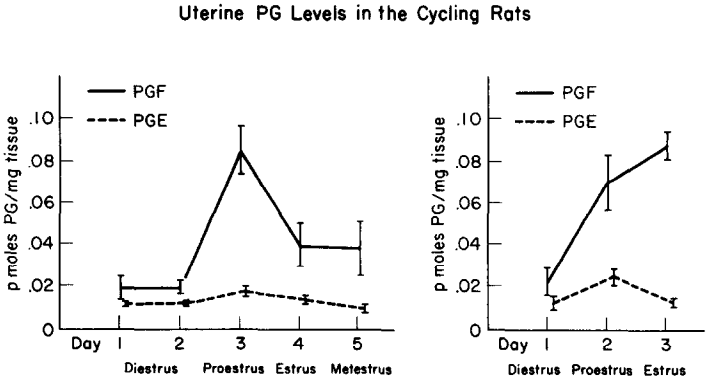

Fig. 4. Prostaglandin levels ($\bar{x}$ ± SEM) in the uterus of the normal cycling rat during the estrus cycle. Prostaglandins were isolated [30] and measured by radioimmunochemical assay [31, 32].

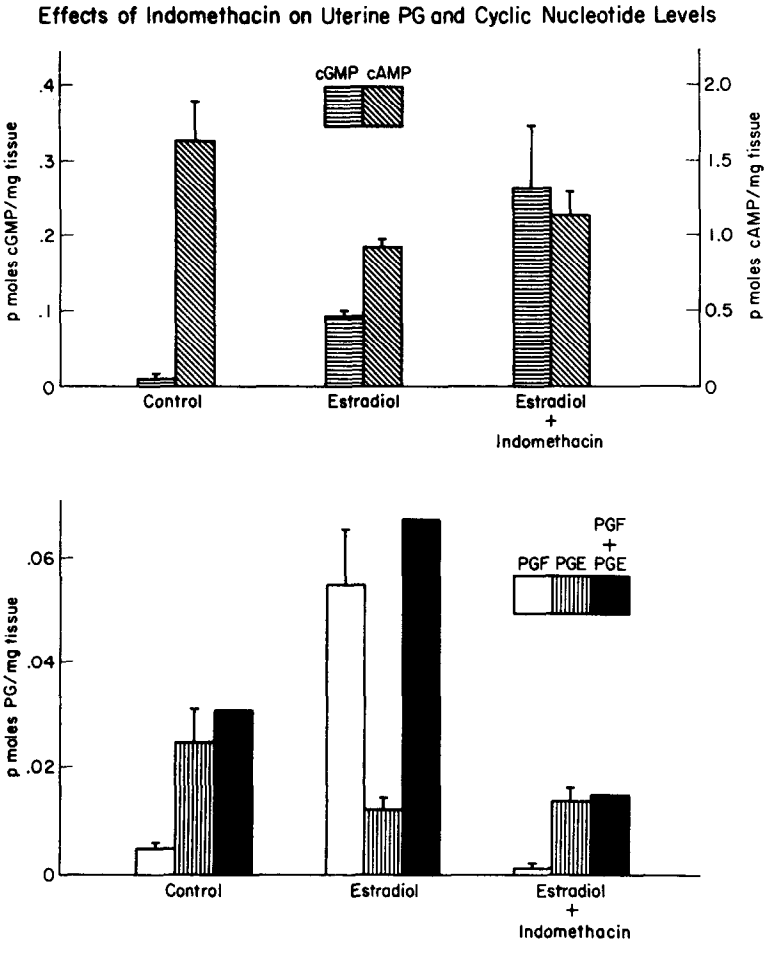

Fig. 6. Ovariectomized rats were treated with estradiol-17β benzoate as described in Fig. 5. Indomethacin (1 mg, in oil, s.c.) was coadministered where indicated. Prostaglandins were isolated [30] and measured by radioimmunochemical assay [31, 32]. Cyclic nucleotides were measured as described [22, 33].

PGF and cyclic GMP production in the uterus are either independent of each other or the rise of PGFs is secondary to cyclic GMP formation. Thus, it is clear that the role of this nucleotide in estrogen action is markedly different from that in the contractile response, where formation of the nucleotide appears to be secondary to PGF action.

The question now arises concerning the manner by which estrogen regulates PG production by the uterus in a specific manner. It is generally accepted that substrate availability (*i.e.*, arachidonic and eicosatrienoic acids) is rate limiting in the formation of prostaglandins. Addition of arachidonic acid has been shown to initiate PG synthesis in ovarian tissue [26]. Although one cannot question the need for a mechanism by which substrate fatty acids are provided, it is unlikely that the ratio of PGEs/PGFs would be effectively regulated by substrate availability alone. An action of estrogen on the synthetase complex or in regulating cofactor availability would seem necessary to impart such product specificity. With the purpose of resolving this issue, we examined microsomes of rat uterine homogenates for their ability to synthesize prostaglandins in the presence of added arachidonic acid. In such a protocol wherein the unknown variables, endogenous substrate precursor acids, would no longer be a complicating factor, the

Table 3. The microsomal fraction of uteri from normal cycling rats was evaluated for the ability to convert 3×10^{-5}M arachidonic acid to prostaglandins. Cofactor concentrations, buffer and incubation conditions were as described [30]. Prostaglandins were isolated [30] and analyzed by radioimmunochemical assay [31, 32]. The difference between the 0-time (endogenous PGs) and the 30-min levels (newly synthesized PGs) represents PG synthetase activity

Stage Of Cycle	Time (min.)	pg PGF per mg protein	pg PGE per mg protein	PGF PGE
	0	490	995	--
Diestrus	30	741	1650	--
	Δ	251	655	0.38
	0	849	921	--
Proestrus	30	1813	1312	--
	Δ	964	391	2.47
	0	1137	867	--
Estrus	30	2933	1103	--
	Δ	1796	236	7.61

prostaglandins formed would be a better measure of synthetase activity. On the basis of three individual experiments, one of which is represented by Table 3, it is evident that the microsomal fraction obtained from the uterus of the cycling rat has an increased ability to synthesize $PGF_{2\alpha}$ from added arachidonic acid, beginning at proestrus to reach a maximum value at estrus. These prostaglandin measurements, in which the increments reflect newly synthesized prostaglandins, also reveal that there is a decreased ability to form PGEs when PGF levels are high. This results in a 20-fold alteration of the ratio PGF/PGE in favor of PGF at estrus compared to diestrus. From these data, it is possible to conclude that the higher levels of PGFs observed in the rat uterus at proestrus and estrus, although necessarily dependent upon endogenous substrate PG-precursor acid availability, are not regulated primarily in this manner. Rather, the nature of the prostaglandin synthetase complex is central to and controls, as a consequence of estrogen action and progesterone counter action, the nature of the prostaglandins formed. In view of the increased prostaglandin synthetase activity as a consequence of estrogen action (data not shown), it is tempting to speculate that this effect is the consequence of induction of specific enzymes, in particular those involved with the formation of F-prostaglandins. However, activation of the enzyme(s) or alteration of cofactors could also be determinants.

As discussed earlier in this paper, there is an increasing body of evidence to show that PGE and PGF can act upon individually unique receptors to trigger entirely different events. In such a scheme the nature of the events triggered would necessarily be dependent upon the type and amount of prostaglandins, the number of receptors available (*i.e.*, PGE vs PGF), or both. Thus, if estrogen produced by the ovary is capable of regulating the synthesis of these potent biologically active lipids in target tissue, in this instance the uterus, a high degree of directive action would be required for full expression of the action of this steroid hormone. This, in fact, appears to be the case, since an early effect of estrogen upon the uterus is to cause a rapid increase in blood flow [27]. There is much evidence to the effect that this action of estrogen is prostaglandin related, and studies in the gravid uterus implicate the PGEs [28] as those involved with this phenomenon. On the other hand, $PGF_{2\alpha}$ has been identified as the luteolytic agent produced by the uterus to cause regression of the corpus luteum in the ovary [29]. Thus, the data presented in this report, demonstrating the ability of estrogen to control uterine prostaglandin synthesis in an orderly and highly directed manner, is consistent with the biological actions of this steroid.

In summation, it is evident from the present studies that estrogen is capable of regulating the synthesis of prostaglandins both qualitatively and quantitatively in target tissue. Contrary to the current concept that availability of prostaglandin precursor acids is the sole factor in regulating prostaglandin levels, the data reported here offer the first evidence to show that prostaglandin synthetase is subject to hormonal control in a highly specific manner. In these actions of estrogen it is evident that prostaglandins are essential for the full expression of the action of this steroid hormone.

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