RNA Polymerase Activity and Uterine Growth: Differential Stimulation by Estradiol, Estriol, and Nafoxidine

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ABSTRACT: We have shown previously that estradiol, estriol, and Nafoxidine (Upjohn 11, 100A) have differential effects on uterine growth and that these effects are associated with the retention of the estrogen receptor by the nucleus. In order to examine these relationships further, we have studied the effect of these hormones on endogenous nuclear RNA polymerase I and II in the immature rat uterus. All three compounds caused a rapid elevation in polymerase II activity that reached a peak by the first hour and declined to almost control levels by 2 h after the injection. This transient peak in polymerase II activity was followed by a second elevation by the fourth hour in estradiol- and Nafoxidine-treated animals which was not observed in estriol-treated rats. The activity of polymerase I increased monotonically to very high levels by 4 h and was maintained 12 h or longer in estradiol- and Nafoxidine-treated animals. A similar elevation was observed in estriol-treated rats but the activity declined very rapidly to control levels by 12 h. The second elevation in polymerase II activity and the sustained stimulation of polymerase I activity were correlated with the stimulation of true uterine growth. These data confirm our previous suggestion that long-term nuclear retention of the receptor is a requirement for true uterine growth and suggest that an obligatory response in the production of true growth is the stimulation of a second rise in polymerase II activity and an elevated and sustained activity of polymerase I.

The primary event in the mechanism of action of estrogen is generally considered to be the binding of estrogen by a target-tissue-specific cytoplasmic macromolecule (estrogen receptor) to form a receptor–estrogen complex. The cytoplasmic receptor–estrogen complex undergoes rapid translocation to the nucleus of the target cell where it probably interacts with chromatin acceptor sites (Gorski et al., 1968; Jensen and DeSombre, 1974; O'Malley and Means, 1974). The interaction of receptor–estrogen complex with acceptor sites in the nucleus is considered to be of pivotal importance in the initiation of specific RNA synthetic events which ultimately result in estrogen-induced responses (Hamilton, 1968; Clark et al., 1973a; O'Malley and Means, 1974).

Estradiol administration to either immature or ovariectomized animals increases RNA synthesis in the uterus (Hamilton et al., 1971; Knowler and Smellie, 1971). Quantitatively these changes have been most marked in rRNA and are measurable within 4–6 h following hormone administration (Hamilton et al., 1968; Hamilton, 1968; Billing et al., 1968). Recently several groups have reported an early increase in very high molecular weight RNA (DNA-like) and have suggested that the marked increase in total RNA which follows may be dependent on the early appearance of this RNA (Knowler and Smellie, 1971; Luck and Hamilton, 1972; Borthwick and Smellie, 1975). Complementary to these results on high molecular weight RNA has been the observation that estradiol treatment results in a comparably early increase in endogenous nuclear RNA polymerase II activity which is followed at later times after estradiol administration (2–4 h) by an increase in the activity of RNA polymerase I and a second rise in RNA polymerase II activity (Glasser et al., 1972; Borthwick and Smellie, 1975).

In addition to the stimulation of RNA synthesis, estradiol elicits a vast number of other uterine responses within 0–6 h of its administration (Hamilton, 1968; Szego, 1971). We have shown that estriol is as effective as estradiol in stimulating transient water imbibition, glucose metabolism, and RNA polymerase activity yet estriol does not produce true uterine growth (Anderson et al., 1972a, 1975; Clark et al., 1974). Thus, it is clear that the mere stimulation of early uterine events does not necessarily lead to true uterine growth. We have suggested that the failure of estriol to cause true uterine growth is due to the rapid loss of the receptor–estriol complex from nuclear acceptor sites in comparison to the longer retention time of the receptor–estradiol complex (Anderson et al., 1972a, 1973, 1975). Long-term nuclear retention may be required for sustained stimulation of nuclear events that are essential for the production of true uterine growth. The retention of the estrogen receptor for long periods of time has also been correlated with sustained uterine growth in Nafoxidine-treated animals (Clark et al., 1973b).

In order to examine further the relationships between nuclear retention of the estrogen receptor and uterine growth, we have measured the effects of estradiol, estriol, and Nafoxidine on the activity of RNA polymerases I and II in the immature rat uterus.

Materials and Methods

Animals. Immature female rats of the Sprague-Dawley Strain (21–23 days old) were obtained from Texas Inbred Mouse Co., Houston, Texas. Animals were kept in a controlled environment of 72 °F with a constant relative humidity and a light–dark cycle of 12 h of each with the light cycle starting at 7 a.m. Food and water were provided ad lib. Estradiol and estriol were prepared in 0.9% NaCl which contained 1% ethanol and Nafoxidine was solubilized in distilled water. Either 0.5 ml of vehicle, estradiol (1.0 μg), estriol (1.0 μg), or Nafoxidine (100 μg) was administered

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subcutaneously at various times prior to sacrifice. Animals were sacrificed by cervical dislocation, and uteri were rapidly removed, stripped of adhering fat, weighed, and placed in an ice-cold solution of 0.9% NaCl-5 mM EGTA.[1]

Isolation of Nuclei. Nuclei were isolated by modifications of the hexylene glycol procedure (Wray and Stubblefield, 1970; Conn and O'Malley, 1975). All procedures were performed at 4 °C. Uteri were removed from the NaCl-EGTA solution, drained, and placed in 1–2 volumes of ice-cold homogenization buffer (1.0 M hexylene glycol, 100 μM $MgCl_2$, 5 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM Pipes, pH 7.5). The tissue was finely minced with iris scissors and 13–19 volumes of ice-cold homogenization buffer were added. The minced tissue was homogenized in a Polytron Pt-10 (Brinkman Instruments) at a setting of 4.2 for three 15–20-s bursts with cooling between homogenization periods. The homogenate was centrifuged at 1500*g* in a Beckman JS-13 rotor for 5 min. The pellet was resuspended in 15 volumes of fresh homogenization buffer by a 10-s burst of the Polytron and the resulting suspension was recentrifuged as above. The pellet from the second centrifugation was drained and resuspended in 10–12 ml of 1.9 M sucrose-TKM (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 mM KCl), transferred to 15-ml Corex centrifuge tubes, and underlayed with 2–3 ml of the same solution. Nuclei were pelleted by centrifugation at 15 000*g* (average) for 50 min in a Beckman JS-13 rotor. Nuclear pellets were gently resuspended in 2–3 ml of TGM buffer (50 mM Tris-HCl, pH 8.0, 25% glycerol, 1 mM $MgCl_2$) with the aid of a Dounce homogenizer equipped with a loose fitting pestle. Nuclei were immediately assayed for endogenous RNA polymerase activities.

RNA Polymerase Assay. Endogenous nuclear RNA polymerase activities were measured by the addition of an aliquot of nuclei (usually 10–30 μg of DNA) to a tube which contained 12.5 μmol of Tris-HCl (pH 8.0), 0.5 μmol of $MgCl_2$, 0.25 μmol of $MnCl_2$, 12.5 μmol of $(NH_4)_2SO_4$, 0.25 μmol of dithiothreitol, 0.025 μmol each of ATP, CTP, and GTP, 0.0025 μmol unlabeled UTP, and 2.5 μCi of [^{3}H]UTP (14–16 Ci/mmol) in a final volume of 250 μl. Some reactions also contained 0.2 μg of α-amanitin. Reactions were incubated at 25 °C for 10 min, terminated by the addition of 2 ml of ice-cold 10% Cl_3CCOOH–1% $Na_4P_2O_7$, and left on ice for 15–30 min. Precipitates were collected on Millipore filters (type HA, pore size 0.45 μm) and washed with 20–30 ml of cold 5% Cl_3CCOOH–1% $Na_4P_2O_7$, and the filters were transferred to a solution of 1 M KCl–5% Cl_3CCOOH–1% $Na_4P_2O_7$ in which they were soaked for 10–15 min. Filters were drained, dried under infrared lamps, transferred to mini-scintillation vials which contained 5.0 ml of scintillation fluid (4.0 g of PPO, 50.0 mg of POPOP/l. of toluene), and were counted in a Beckman liquid scintillation counter at 22% efficiency. One picomole of [^{3}H]UMP incorporated represents 540 cpm under these assay conditions.

DNA Determination. DNA was estimated by the diphenylamine method of Burton (1956).

Chemicals. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals, [^{3}H]UTP [5-^{3}H] from Schwarz/Mann, and [^{3}H]estradiol-17β [6,7-^{3}H(N)] from New England Nuclear. Estradiol-17β and estriol were obtained from Sigma Chemicals, while Nafoxidine was a gift of the Upjohn Co. All other chemicals were of reagent grade.

Results

Nuclei Isolation and RNA Polymerase Assays. The following modifications were made in the hexylene glycol procedure of Conn and O'Malley (1975): the hexylene glycol concentration was increased from 0.5 to 1.0 M to effect more thorough-tissue dispersal; EGTA was added to the homogenization media to chelate interstitial Ca^{2+} which consequently reduced tissue clumping and released more nuclei; Mg^{2+} was substituted for Ca^{2+} as a divalent cation to prevent nuclear rupture; 2-mercaptoethanol was included to stabilize endogenous RNA polymerase activity.

These modifications of the hexylene glycol procedure allowed adaptation of the procedure to 300–600 mg of uterine tissue and routinely yielded 30–50% recoveries of the DNA present in initial homogenates as purified nuclei. Purity of nuclei was assayed by phase-contrast microscopy which revealed few cytoplasmic tags and little nuclear clumping. A typical preparation contained two morphologically distinct classes of nuclei which probably represent nuclei from both myometrium and endometrium. The uterine nuclei prepared by this procedure retain high levels of RNA polymerase I and II activities.

Conditions for RNA synthesis in isolated nuclei were chosen such that both RNA polymerase I and II activities were expressed (Reeder and Roeder, 1972; Marzluff et al., 1973). RNA polymerase II activity was determined by its sensitivity to low concentrations of α-amanitin (0.8 μg/ml). The remaining activity was defined as RNA polymerase I, although it actually consists of at least two activities, RNA polymerase I and RNA polymerase III. RNA polymerase III appears to be a minor component of the α-amanitin resistant activity in nuclei isolated by methods described in this communication and was therefore not measured separately (J. W. Hardin, unpublished observation). The conditions employed for measurement of endogenous RNA polymerase activities in this study do not allow the measurement of absolute amounts of a particular RNA polymerase species but rather reflect changes in the level of activity of the various classes of enzyme present in nuclei.

In order to compare the levels of RNA polymerase activities present in nuclei isolated from different groups of animals, it was necessary to demonstrate initial velocity kinetic conditions existed for assay conditions with respect to time of incubation and to quantity of nuclei added to reactions. RNA polymerase I and II activities are linear for at least 20–30 min at 25 °C, and both species of enzyme exhibited linear incorporation of nucleotides into RNA over a wide range of increasing DNA concentrations added as nuclei to reactions.

RNA Polymerase Activities in Isolated Nuclei Following a Single Injection of Hormone. Single injections of estradiol, estriol, or Nafoxidine cause a transient rise in endogenous nuclear RNA polymerase II activity which reaches a peak 1 h after hormone treatment (Figure 1A). The greatest response at this time is elicited by estradiol and estriol which cause a significant increase in enzymatic activity as early as 30 min after hormone treatment. Nafoxidine has

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; hexylene glycol, 2-methyl-2,4-pentanediol; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Cl_3CCOOH, trichloroacetic acid; SEM, standard error of the mean; SD, standard deviation. TKM buffer, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 mM KCl; TGM buffer, 50 mM Tris-HCl, pH 8.0, 25% glycerol, 1 mM $MgCl_2$; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

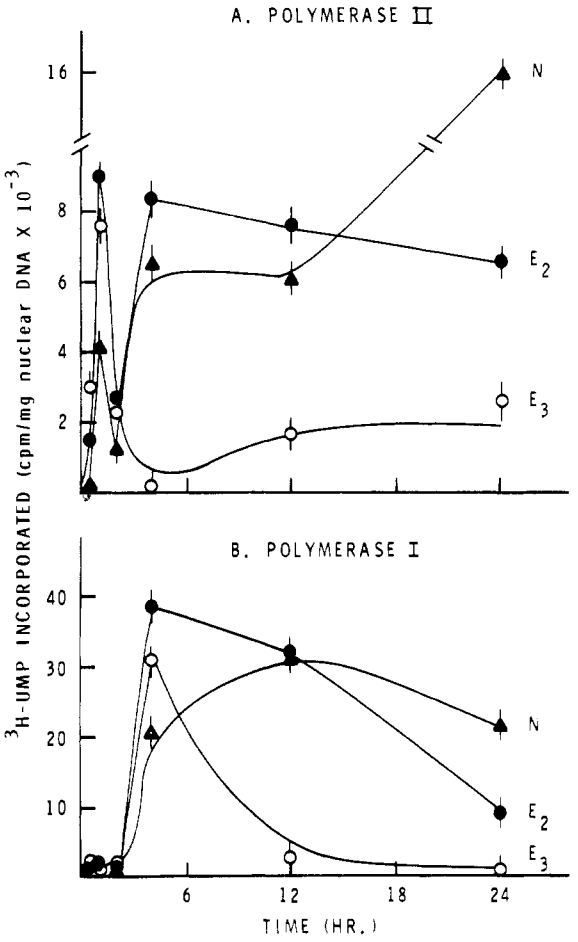

FIGURE 1: Time course of nuclear RNA polymerase I and II following hormone injections. Animals were injected with either estradiol, estriol, or Nafoxidine as described in the text. At indicated times, uteri were removed, nuclei isolated, and endogenous RNA polymerase activities determined. All points represent six to nine determinations ±SEM. (A) RNA polymerase II activity; (B) RNA polymerase I activity. E_2 (●); E_3 (○); N (▲).

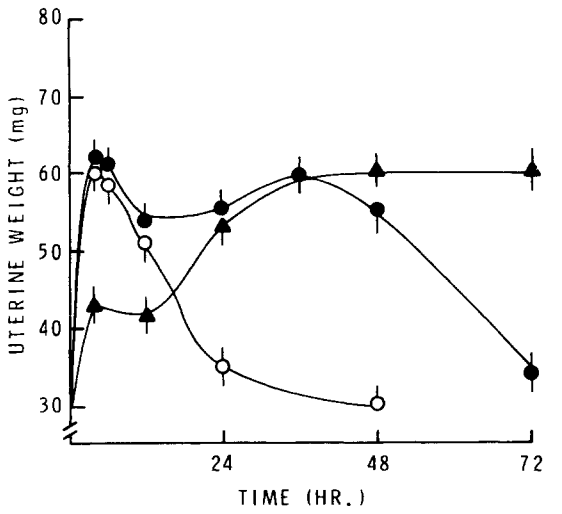

FIGURE 2: Effects of a single injection of estradiol, estriol, or Nafoxidine on uterine wet weight. Rats were injected with hormones as described in the text. At various times following injection, rats were sacrificed and uterine wet weights determined. E_2 (●); E_3 (○); N (▲).

Table I: The Content of Uterine DNA, RNA, and Protein 24 Hours after a Single Injection of Various Estrogens and "Anti-estrogens".

Treatment	DNA (μg/uterus ± SD)	RNA (μg/uterus ± SD)	Protein (mg/uterus ± SD)
Saline	343 ± 28	144 ± 35	3.17 ± 0.24
Estradiol	358 ± 19	490 ± 25	5.69 ± 0.31
Estriol	408 ± 40	175 ± 34	3.20 ± 0.21
Nafoxidine	383 ± 22	408 ± 42	6.01 ± 0.41

little, if any, effect at this very early time and evokes less of a response at 1 h than do the other two compounds. This decreased response is correlated with the slower rate of nuclear accumulation of the Nafoxidine–receptor complex (Clark et al., 1973b).

The activity of polymerase II declines dramatically by 2 h after the injections in all three groups. This decline is followed by a second elevation in activity in estradiol-treated animals which reaches a maximum by 4 h (Figure 1A). A similar elevation in polymerase II activity in Nafoxidine-treated animals is followed by another large late increase which occurs between 12 and 24 h after the injection (Figure 1A). A second elevation in enzyme activity was not observed in the estriol-treated animals and the activity of polymerase II remained low between 4 and 24 h.

Following the early transient increase in uterine nuclear RNA polymerase II activity, an increase in RNA polymerase I activity occurred in all hormone groups by 4 h (Figure 1B). Estriol caused a transient rise in this activity which was characterized by a regression phase between 4 and 12 h (Figure 1B). Estradiol treatment results in a marked elevation in RNA polymerase I activity by 4 h which is followed by a slow decline in activity to values that remain significantly above those of the control at 24 h. Nafoxidine treatment results in a similar elevation of endogenous RNA polymerase I activity by 4 h which is maintained at levels as high or higher than those induced by estradiol (Figure 1B).

Effects of a Single Injection of Estradiol, Estriol, or Nafoxidine on Long-Term Uterine Growth. Estradiol and estriol cause dramatic increases in uterine weight by 4 h (Figure 2). This increase is followed by a loss of water which results in a rapid decline in uterine weight in estriol-treated animals to near control levels by 24 h. The uterus of estradiol-treated animals experiences a small transient weight loss and then continues to grow. This second growth phase, which constitutes true uterine growth (see below and Table I), reaches a maximum by 36 h. Uterine regression follows rapidly and the weight declines to control levels by 72 h. The effect of Nafoxidine on uterine wet weight by 4 h was not as great as that of estradiol or estriol; however, Nafoxidine caused elevated uterine weights by 24 and 48 h which were sustained at 72 h (Clark et al., 1973b).

Estradiol and Nafoxidine cause significant elevations in the content of RNA and protein in the uterus by 24 h after the injections (Table I). The elevations are in marked contrast to the failure of estriol to elicit any significant increase in these parameters. DNA content was unaffected in all groups by 24 h, though changes can be observed in 36–48 h (Mueller et al., 1958).

Discussion

The results of this study show that estradiol and estriol are of equal ability in stimulating a rapid increase in RNA polymerase II activity which reaches a peak 1 h after injection. This is closely correlated with the ability of these hormones to cause nuclear translocation and accumulation of the receptor–estrogen complex (Anderson et al., 1972a). On

the other hand, Nafoxidine, which results in a slower accumulation of receptor by the nucleus (Clark et al., 1973b), produces a peak of polymerase II activity which is approximately 50% less than that produced by either estradiol or estriol. The second elevation of polymerase II activity which was observed by 4 h in estradiol- and Nafoxidine-treated animals was not observed in estriol-treated animals. The failure of estriol to stimulate this second elevation in RNA polymerase II activity may be one of the factors involved in the inability of estriol to stimulate true uterine growth.

We have proposed, as a working hypothesis, that the estrogen receptor must remain bound in the nucleus for approximately 6 h or longer in order to simulate true uterine growth (Anderson et al., 1972a, 1973; Clark et al., 1973a, 1976) and have shown that the inability of estriol to cause true uterine growth is correlated with short-term nuclear retention (Anderson, 1972a). Thus, following a single injection of estriol, the receptor does not remain in the nucleus for a sufficient period of time and, although it stimulates an early increase in RNA polymerase activity, it does not produce true uterine growth (Figure 2, Table I). Nafoxidine, on the other hand, causes long-term retention of the receptor by the nucleus and sustained growth of the uterus (Figure 2, Table I; Clark et al., 1973b). This effect of Nafoxidine has been shown to be very long, lasting up to 19 days in the immature rat (Clark et al., 1973b).

The elevation of polymerase I activity which was stimulated by all three compounds was maintained for much longer periods of time by treatment with estradiol and Nafoxidine. The rapid decline in polymerase I activity in the estriol-treated animals may result from the short-term residency of the receptor–estriol complex in the nucleus (Figure 1; Anderson et al., 1972a). These data demonstrate that the secondary rise in polymerase II activity, as well as the duration and magnitude of the increase in polymerase I activity, are correlated with the retention of the nuclear estrogen receptor–hormone complex and the production of true uterine growth.

The early (30 min) estrogen-dependent rise in uterine endogenous nuclear RNA polymerase II activity was first described by Glasser et al. (1972) in the mature ovariectomized rat and subsequently confirmed in the immature rat (these data) and immature rabbit (Borthwick and Smellie, 1975). The initial rise in polymerase II activity was blocked by the prior in vivo administration of α-amanitin (Glasser et al., 1972) and actinomycin D but not by cycloheximide (Means and Hamilton, 1966; Glasser and Spelsberg, 1973). The secondary rise in polymerase II activity and the increase in polymerase I activity were sensitive to all three inhibitors (Glasser and Spelsberg, 1973). From these data the suggestion was first derived that estradiol evokes an early increase in polymerase II activity. This early stimulation of putative mRNA synthesis (Knowler and Smellie, 1971; Glasser et al., 1972; Borthwick and Smellie, 1975) may be involved in the subsequent synthesis of uterine proteins involved in the later increase of both polymerases and, in turn, other proliferative changes which characterize the uterotrophic action of estrogen. A similar suggestion was made by Raynaud-Jammet et al. (1972) who found an RNA product whose synthesis, which was sensitive to α-amanitin, appeared necessary for the increase in RNA polymerase I activity which followed estradiol administration.

Our present results would indicate, however, that the action of estrogen on RNA synthesis may be more complex and difficult to interpret than simply relating an early effect in RNA polymerase II activity to the synthesis of a product that modulates the later increases in the activities of the polymerases. Thus, an injection of estriol to immature rats, which evokes as great an increase in polymerase II activity at 30–60 min as does estradiol (Figure 1a), fails to produce the secondary increase in polymerase II activity and evokes only a transient increase in RNA polymerase I activity (Figure 1b). The failure of estriol to continue to stimulate RNA synthesis is reflected in both the disappearance of the receptor–estriol complex from the nucleus and the failure of the hormone to stimulate true uterine growth. Nafoxidine in comparison causes only a small early increase in polymerase II activity but produces a substantial and sustained increase in polymerase I and a large secondary stimulation in polymerase II activity as well as true uterine growth. Both of these results agree with our findings (Anderson et al., 1972a, 1975) which demonstrated that the estrogen receptor–hormone complex must be retained in target cell nuclei for some relatively finite critical period of time to elicit true uterine growth.

The precise mechanisms by which long-term retention of nuclear estrogen receptor–hormone complex stimulates both RNA synthesis and uterine growth is not clear. However, results from other steroid hormone systems would suggest the most likely possibility is some modification of chromatin by the steroid receptor–hormone complex. It has been shown recently that estrogen stimulation of the chick oviduct brings about an increase in the number of rifamycin resistant *Escherichia coli* RNA polymerase initiation sites present in oviduct chromatin (Schwartz et al., 1975) and that receptor binding by the nucleus is correlated with this increase in RNA polymerase initiation sites (Kalimi et al., 1976; Tsai et al., 1975). With respect to RNA synthesis, Borthwick and Smellie (1975) have been unable to show any changes in the amount of extractable RNA polymerase molecules from immature rabbit uterus following stimulation of RNA synthesis by in vivo estrogen administration. In contrast, however, it has recently been shown that the steroid hormone, 1α,25-dihydroxy-vitamin D_3, does cause a rapid increase in the amount of extractable RNA polymerase II activity from chick intestinal cells (Zerwekh et al., 1974).

The stimulation of true uterine growth is not characteristic of every estrogen. Of the varied multiple effects which characterize the biological action of each estrogen, only certain correlated sets of events stimulate true uterine growth and are an indication of uterotrophic estrogen. Thus, stimulation of nuclear receptor binding and RNA synthesis, per se, will not cause true uterine growth. Rather the estrogen receptor must be retained in the nucleus for some critical period. Retention of the receptor is correlated with a secondary increase and sustained activation in RNA polymerase II activity and a sustained elevation in RNA polymerase I activity. These effects on RNA polymerase I and II appear necessary for true uterine growth.

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