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ONTOGENY OF THE ESTROGEN INDUCIBILITY OF UTERINE PEROXIDASE

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SUMMARY

The estrogen inducibility of the enzyme, peroxidase (donor: hydrogen-peroxidase oxidoreductase, EC 1.11.1.7), in the uterus of the 3–22 day old rat was studied and compared to the ability of estrogen to stimulate increases in uterine weight. Neither estradiol nor diethylstilbestrol were able to effect significant increases in uterine weight or peroxidase content in 4 or 7 day old rats. Around 11–13 days of age the uterus becomes sensitive to both estrogens as indicated by both significant estrogen stimulation of uterine weight and induction of uterine peroxidase 20 h after either estrogen. Since diethylstilbestrol, which does not readily bind to α-fetoprotein, is also incapable of stimulating uterine weight or inducing peroxidase in the 4 and 7 day old animal it is likely that the insensitivity of the early postnatal uterus to estrogen is not due to the high circulating levels of α-fetoprotein. At the same age that the uterus becomes sensitive to the effects of estrogen it also becomes sensitive to progesterone inhibition of the estrogen-stimulation. These studies indicate that the inducibility of uterine peroxidase correlates very well with the estrogen stimulation of uterine weight in the postnatal rat and that the ontogeny of hormonal sensitivity of the uterus appears rather abruptly, within a period of several days.

INTRODUCTION

The significant advances in our understanding of the mechanism of action of steroid hormones in general, and estrogens in particular, over the last two decades has resulted from studies using a variety of approaches to steroid hormone studies. Investigations of the interaction of estrogen with target tissues *in vivo* and *in vitro* led to the recognition of the multi-step interaction pathway of estrogen with its target tissues [1, 2]. On entering the target cell, estrogen rapidly associates with its cytoplasmic receptor protein to form a complex which readily undergoes a temperature-dependent, steroid-specific activation. The activated complex, unlike its precursor, readily enters the nucleus and associates with cellular chromatin [3]. Studies of the biochemical effects of estrogen have identified an early effect of estrogen on uterine RNA synthesis *in vivo* [4–6]. More recent investigations indicate that the activated estrogen-receptor complex can effect a target-tissue specific increase in RNA polymerase I activity *in vitro* [7]. Although a number of specific responses to estrogen in the uterus have been investigated, the details of the relationship between the estrogen-receptor interaction and the effect of estrogen on the specific biochemical events which lead to the growth and function of this tissue are as yet unknown [8]. However, studies with human breast cancer tissue suggest that the cytosol estrogen receptor is an essential, but not necessarily sufficient, condition for biologic dependence on the hormone [9, 10].

The developing uterus appears to be a useful model to study the acquisition of estrogen responsiveness as correlated with the estrogen receptor interaction. In a series of reports by Kaye, Somjen and Lindner[11–13], and the data of Clark and Gorski[14] it was concluded that while the neonatal rat uterus contains appreciable amounts of estrogen receptor, the uterus of the rat is insensitive to stimulation of growth until after about 10 days of age [15].

Previous investigations in our laboratory [16–19] have led us to propose that the enzyme, peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7), may be a meaningful marker of estrogen-dependent growth. Although there is no evidence to suggest that peroxidase induction is a requirement for uterine growth and indeed its function has not yet been clarified, nonetheless estrogen-dependent increases in peroxidase closely parallel tissue growth [18, 19]. It was therefore of interest to elucidate the ontogeny of the estrogen inducibility of uterine peroxidase as related to the estrogen stimulation of uterine growth in the developing rat. We report herein that, consistent with its use as a marker, the ontogeny of the peroxidase inducibility and growth responsiveness to estrogen in the postnatal uterus are identical.

MATERIAL AND METHODS

Animals. Sprague–Dawley derived, female lactating rats, each fostering litters of 10 female pups, were obtained from King Animal Laboratories, Inc., Oregon, WI. The animals arrived in our laboratory at least 2 days prior to use and each mother and litter was housed in a separate cage.

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Experimental procedures. The pups in each litter were kept with the foster mother but marked as two groups, providing 5 animals each for the estrogen-treated and control (or estrogen + progesterone treated) groups at each age. Diethylstilbestrol and estradiol-17β (Sigma) were injected at 40 μg per kilogram body weight using 5 or 10 μg per ml solutions of the estrogens in 10% ethanolic saline. For these experiments control animals received injections of vehicle alone. For the study comparing the effect of estradiol alone to the combination of estradiol and progesterone, the injection vehicle used was 10% ethanol in sesame oil and the estradiol (also 40 μg per kilogram body weight) was given alone or combined for a single injection with progesterone (20 mg per kilogram body weight). In all cases the pups were returned to the foster mother after subcutaneous injection of the solutions, until sacrificed by decapitation, 20 h later. The uteri were removed, dissected free of fat and connective tissue and rapidly nicked with a scissors, blotted on filter paper to express fluid and weighed.

Assay procedure. Unless otherwise mentioned all procedures were carried out at 2–4°C. Each uterus was minced with scissors and homogenized at a concentration of 25 mg per ml, or in 1 ml if less than 25 mg, in 10 mM tris, pH 7.2, buffer using a Polytron PT10ST homogenizer. For the 4 day old animals however, where the uteri weighed 3 to 6 mg, the entire group of 5 minced uteri were combined prior to homogenization. Aliquots of the homogenates were removed for DNA analysis and the remainder centrifuged 40 min at 39,000 g. The supernatant fractions, which contain insignificant amounts of peroxidase [18], were discarded and the sediments were rehomogenized with 1 ml of an extraction buffer consisting of 500 mM $CaCl_2$ in 10 mM Tris, pH 7.2, buffer. The peroxidase containing extract was clarified by a 40 min, 39,000 g, centrifugation and assayed by measuring the rate of oxidation of guaiacol at 25°C as previously reported [18], but assay volumes were modified to use the Gilford Model 3401 automated enzyme analyzer while maintaining the previously determined optimal final substrate concentrations of 13 mM guaiacol and 0.3 mM H_2O_2. Linear initial rates were converted to enzyme units, defined as the amount of enzyme which produced an increase of 1 absorbance unit at 470 nm per minute at 25°C.

RESULTS

The effect of administered estradiol on the wet weight and peroxidase content of uteri of immature female rats of various ages is shown in Fig. 1. With rats up to 11 days of age there was no significant effect of estradiol on either of these parameters. At 13 days of age a single injection of estradiol brought about a significant increase in uterine weight (50% greater than control) and a dramatic induction of uterine peroxidase. Similar responses to estradiol were found with pups between 13 and 22 days of age, indicative of a rapid and complete onset of uterine responsiveness at about 13 days of age.

Since the serum of very young rats has high concentrations of α-fetoprotein, a protein known to have a high affinity for estradiol, one might ascribe the lack of uterine response to estradiol in the 4 to 10 day old rat to a lower tissue availability of estradiol resulting from its association with α-fetoprotein in the blood [20]. Therefore, a similar study was performed using the synthetic estrogen, DES, which does not readily bind to α-fetoprotein. The results of this study are shown in Fig. 2. It can be seen that no uterine response to DES was observed in the 4 and 7 day old rats. The DES stimulated increase in uterine weight and induction of uterine peroxidase occurred in the 11 day old rat and both parameters showed estrogen stimulation in all rats 11 days or older.

Since the ontogeny of uterine responsiveness to estrogen appeared distinctly and abruptly as a function of neonatal age it was of interest to determine whether sensitivity of the uterus to modulation by other hormones would appear at a similar time. Progesterone, a known antagonist to the action of estrogen in the uterus [21], was administered along with estrogen to determine the neonatal age at which progesterone antagonism could be observed. As shown in Fig. 3, by 14 days of age, the uterus of the developing rat is sensitive to both estrogen stimulation and the progesterone antagonism thereof. Treatment with progesterone at the same time as estradiol prevented the estradiol-dependent increase in peroxidase and diminished the estrogen-dependent increase in uterine wet weight. With rats of 12 days

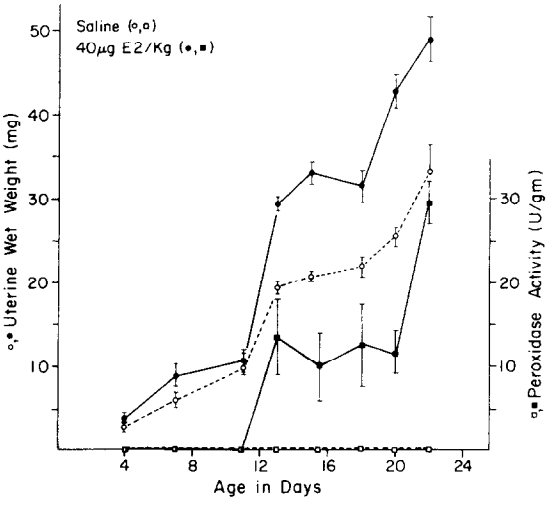

Fig. 1. Effect of estradiol on uterine wet weight and uterine peroxidase concentration in the postnatal female rat. Estradiol-17β (40 μg/Kg body weight, closed symbols, solid lines) or saline (open symbols, dashed lines) was administered subcutaneously to 5 pups each of the same age from the same foster mother fed litter and the pups were sacrificed 20 h later for assay of uterine wet weight (circles) and uterine peroxidase (squares). Values presented as mean ± S.E.M.

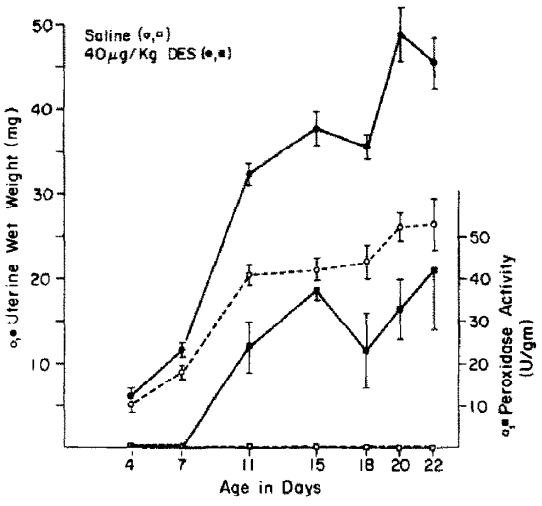

Fig. 2. Effect of diethylstilbestrol (DES) on uterine weight and uterine peroxidase concentration in the postnatal female rat. DES (40 μg/Kg body weight, closed symbols, solid lines) or saline (open symbols, dashed lines) was administered subcutaneously to 5 pups each of the same age from the same foster mother fed litter and the pups were sacrificed 20 h later for assay of uterine wet weight (circles) and uterine peroxidase (squares). Values presented as mean ± S.E.M.

of age, despite the relatively small amount of peroxidase induced on the administration of estradiol alone, there was a significant inhibition of peroxidase induction in the animals treated with progesterone and estradiol ($P = 0.05$ by Mann–Whitney rank sum statistics). Concomitantly, progesterone also inhibited the uterine weight increase due to estrogen ($P = 0.025$). Interestingly, in the 12-day old rats the concentration of peroxidase correlated ($r = 0.75$ $P = 0.02$) with the uterine weights as the larger uteri in general had higher concentration of peroxidase even though the overall response of these uteri was considerably less than was that of the 14-day old animals where the response was striking.

DISCUSSION

Studies of the estrogen sensitivity of the postnatal rat uterus consistently show that this tissue is able to respond to hormone significantly before puberty. With most parameters no significant estrogen stimulation of the uterus is seen before 10 days of age but by 15 days of age administration of a single dose of estradiol leads to an increase in uterine weight as well as in uterine protein and RNA content [15]. However as regards DNA synthesis in the uterus an effect of estradiol is only seen in the rat 20 days of age or older [12]. Regarding specific cellular constituents, Katzenellenbogen reported an estrogen-dependent increase in phosphorylation of 2-deoxyglucose in uteri of 12–15 day old rats *in vitro* [22].

Unlike the foregoing responses, it appears that the estrogen-induced uterine protein, IP, first reported by Notides and Gorski[23], may be stimulated by estrogen prior to day 10. Katzenellenbogen and Greger[22] found that IP synthesis, on a per cell basis, may be maximally induced in the uterus of the 5 day old, estrogen-treated rat based on the observation that the ratio of IP synthesis to DNA remains constant in the uteri of estrogen-treated rats of 5–18 days of age. Walker *et al.*[24] found a minimal response of IP in the 5 day old rat. They reported that by day 9–10 the IP response was maximal. These data therefore suggest that the inducibility of IP precedes the majority of uterine responses to estrogen in the developing uterus, and hence, while a possible key intermediate protein [25], IP would not appear to be closely correlated with the growth responsiveness of a tissue to estrogen.

The data presented here indicate that the estrogen inducibility of uterine peroxidase in the postnatal rat correlates very well with the estrogen stimulation of uterine weight. Furthermore, there appears to be a rapid onset of the uterine responsiveness to hormone, occurring between 11 and 13 days. Although the initial response to DES appeared in 11 day old rats while the first age of response in the estradiol study was in the 13 day old rat, this difference is believed to reflect animal variability. It is of interest that in both of these experiments the initial responses were seen when the control uterine weights exceeded 19 mg. This might suggest a uterine maturation related to uterine size rather than exact neonatal age. Because of the uncertainties of maternal feeding of pups prior to weaning age (20 days) it is important

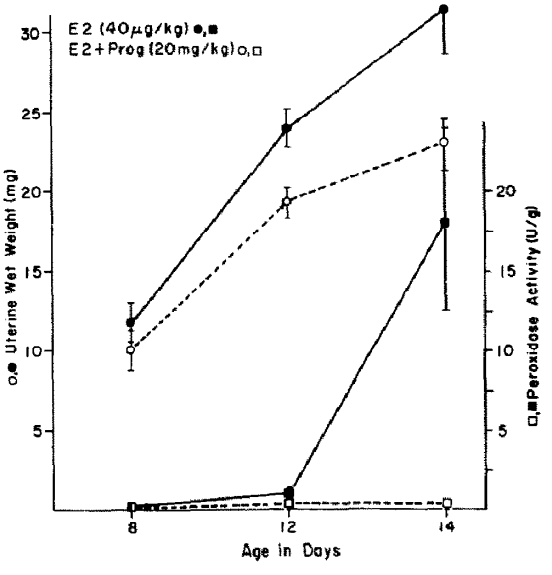

Fig. 3. Progesterone antagonism of estradiol action in the postnatal rat uterus. Estradiol-17β (40 μg/Kg body weight, solid lines, closed symbols) alone or combined with progesterone (20 mg/Kg body weight, dashed lines, open symbols) was administered subcutaneously to 5 pups each of the same age from the same foster mother fed litter. The pups were sacrificed 20 h later for assay of uterine wet weight (circles) and uterine peroxidase (squares). Values presented as mean ± S.E.M.

to control litter size to minimize spurious results. In these studies we used artifically created litters of 10 female pups so that the 5 control and 5 experiment pups of each age were always from the same litter. The results of the study of progesterone antagonism (Fig. 3) suggest that when uterine competence, as measured by sensitivity to estrogen stimulation of weight gain and induction of peroxidase, is acquired, such competence includes sensitivity to modulation by other hormones, such as progesterone.

Raynaud[20] has assayed the levels of the estrogen binding proteins (EBP) in the serum of neonatal rats and found a 10 fold decrease in the concentration of this parameter between 1 and 17 days of age. He concluded that the high concentration of EBP in the 5 day old rat was the reason the uterus did not respond to estradiol but did respond to R2858, a synthetic estrogen which has a low affinity for the serum EBP. The reported 24 h uterine wet weight increase to R2858 in the 5 day old rat (increase of 15–30%) was minimal compared to the responses of the 13 day old (68–76%) and 21 day old (100–161%) rats [20]. Our finding that the effects of DES, which like R2858 does not have an appreciable affinity for α-fetoprotein, are essentially the same as those after estradiol would seem to be at disparity with those of Raynaud. However, close inspection of the wet weight curves for uteri after both estradiol (Fig. 1) and DES (Fig. 2) discloses small increases in weight in the 4–7 day old rats and in each case the uterine weights of the estrogen treated rats are at least slightly higher than the controls. It is apparent here, as well as in Raynaud's data, that the magnitude of the estrogen effect seen in the older animals is significantly different. It may be that these small increases simply reflect the hyperemia and water-imbibition seen as an early response to estrogen rather than the tissue growth evident in the full response to estrogen in the competent uterus.

The relation of the ontogeny of biochemical responses to estrogen in the developing uterus and the function of estrogen receptors is not at all clear at this time. Clark and Gorski[14] showed that the uterus of the 1 day old rat contains estrogen receptor and by 5 days the uterine cytoplasmic receptor concentration is about 2/3 that of the 21 day old animal. Since it is clear that excess receptor (over that used by a physiologic dose of estradiol) is present in the 21 day old rat [26] it would appear that the 5 day old rat uterus does not lack a sufficient quantity of receptor for response. Furthermore, the affinity for estrogen and the sedimentation characteristics of the cytosol receptors in the 5, 10 and 22 day old rat uterus are indistinguishable [14]. Finally, the cytosol receptor of the 5–7 day old rat uterus appears to be competent as regards translocation and nuclear uptake [13]. It would therefore appear that one or more steps subsequent to the estrogen-receptor uptake by nuclei are not fully competent in the uterus of the rat before 10 days of age. With the documentation that response of uterine peroxidase appears to reflect the more general growth responsiveness of the postnatal rat uterus to estrogen, one now has a more specific tool to study the details of the ontogeny of the biochemical responsiveness to estrogen and the role of the estrogen-receptor interaction pathway in the development of uterine sensitivity to estrogen.

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