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# STEROID BINDING ALTERATIONS IN TISSUE COMPARTMENTS OF THE VAGINA OF CONTROL AND NEONATALLY DIETHYLSTILBESTROL-TREATED ADULT MICE

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**Summary**—Steroid binding in both the vaginal epithelium and the vaginal fibromuscular wall (FMW) was compared in control and neonatally estrogen-treated mice. Neonatal treatment with a low dose of the estrogen diethylstilbestrol (DES) had no significant effect on adult estrogen binding within the assayed vaginal compartments; however, this treatment caused a 2-fold increase in the level of cytosolic progestin binding in the vaginal FMW over that in vehicle-treated mice. This low neonatal dose did not affect the level of progestin binding in the vaginal epithelium. In contrast, neonatal treatment with a larger dose of DES caused marked increases in cytosolic progestin binding, decreases in cytosolic estrogen binding, and increases in nuclear estrogen binding within the FMW. Furthermore, as a result of the changes in specific binding induced by the neonatal DES treatment, the degree of the estrogen binding within in each tissue shifted from a predominantly cytosolic site to a nuclear one.

## INTRODUCTION

Exposure to the synthetic estrogen diethylstilbestrol (DES) during early prenatal development has been linked to the appearance of clear cell adenocarcinomas in the reproductive tract of young women [1]. Since the earliest appearance of this cancer coincides with the pubertal rise in endogenous hormone secretion, the reproductive tract in the affected individuals may express altered sensitivity or response to this rise in hormone concentration. Much of the human response to prenatal estrogen is mirrored in the mouse response to neonatal estrogen [2], including an altered sensitivity to estrogen in adult tissues [3–8].

In an attempt to find biochemical correlates of this altered responsiveness, steroid receptor levels have been assayed in the reproductive tract and mammary gland of neonatally estrogen-exposed mice and rats [9–11]. In a recent report, mice treated with a neonatal dose of DES sufficient to cause ovary-independent vaginal cornification had significantly decreased levels of vaginal estrogen receptors relative to controls [12]. This decrement in cytosolic estrogen receptors was present 1, 2, 6 and 12 months after DES treatment. Lower neonatal doses of estrogen had little effect. However, these measurements were obtained from whole vagina, raising the possibility that changes induced by neonatal DES exposure could occur in one tissue compartment, but be masked by small or opposing changes in another tissue. This was made clear by the report of Uchima *et al.*[13], which demonstrated that estrogen and progestin receptors are present in both the epithelium and the fibromuscular wall of the mouse vagina. Furthermore, differences could exist in estrogen binding within either the nuclear or cytosolic compartments, which were either unknown or being masked. Answers to these questions could help explain the frequent lack of correlation between the cytosolic estrogen receptor level and the estrogen responsiveness of an organ.

## EXPERIMENTAL

### *Materials*

Medium 199 was purchased from Grand Island Biological (Santa Clara, Calif.); Percoll and Dextran T-70 from Pharmacia Fine Chemicals (Piscataway, N.J.); collagenase CLS III (158 U/mg) from Cooper Biomedical (Malvern, Penn.); [17$\beta$-methoxy-$^3$H]-R2858 (moxestrol, 87 Ci/mmol), [17$\alpha$-methyl-$^3$H]-R5020 (promegestone, 87 Ci/mmol), unlabeled R2858 (17$\alpha$-ethynyl-1,3,5(10)-estratriene-3,17$\beta$-diol), unlabeled R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione), and Aquasol from New England Nuclear (Boston, Mass). Other chemicals were purchased from Sigma Chemical (St Louis, Mo.), unless otherwise stated.

### *Animals*

Female BALB/cCrgl mice were given daily subcutaneous injections of either 0.02 ml sesame oil

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vehicle or diethylstilbestrol (DES) in sesame oil for the first 5 days of life. Two doses of DES were used: $10^{-1}$ $\mu$g/day, which generally results in ovary-independent vaginal cornification, and $10^{-3}$ $\mu$g/day, which generally results in ovary-dependent vaginal cornification [12]. At 30 days of age, all mice were ovariectomized. At 39 days, the mice were killed and the vaginae were removed, placed in medium 199 supplemented with 100 U/ml penicillin and 100 $\mu$g/ml streptomycin, and stored on ice until dissociation. In mice treated with the high dose of DES, only fully cornified vaginae were used (approximately 80% of the vaginae obtained). In mice treated with the lower dose of DES, any cornified vagina encountered was assumed to be the result of ovary-independent cornification and was discarded.

*Tissue dissociation*

Using a technique similar to that of Uchima *et al.*[13], vaginae were first enzymatically treated with 0.13% collagenase in medium 199 containing 0.5% serum albumin (bovine, Fraction V) for 2 h at 37°C, and then physically separated into sheets of pure epithelial and fibromuscular wall (FMW) tissue. These tissues were then applied separately to preformed Percoll gradients and centrifuged at 1085 $g$ for 20 min. After removal from Percoll, the tissue was washed in medium 199, and homogenized with two 10-s bursts of a polytron (PT-10-ST; Brinkmann Instruments, Westbury N.Y.) in 20 vol homogenization buffer (25 mM Tris, 1.5 mM EDTA, 10 mM sodium molybdate, 10 mM monothioglycerol, 10% (v/v) glycerol, pH 7.4). The homogenate was centrifuged for 20 min at 4°C and 1000 $g$. The supernatant was considered to be the cytosolic fraction. The pellet, or nuclear fraction, was washed three times with homogenization buffer and then placed in this buffer containing 0.4 M KCl for 60 min at 4°C to extract nuclear estrogen binding sites bound to chromatin. Both cytosolic and nuclear fractions were centrifuged for 30 min at 4°C at 105,000 $g$. Supernatants were removed, frozen and stored at −60°C. The nuclear pellet was assayed for DNA content using calf thymus DNA as a standard [14].

*Cytosolic steroid binding assays*

The labeled ligands for the determinations of estrogen and progestin binding were [$^3$H]R2858 and [$^3$H]R5020, respectively. Saturating concentrations of radiolabeled ligand were used throughout [10]. In a standard assay, 0.2-ml aliquots of cytosol were incubated with 0.1 ml radiolabeled tracer in buffer B (10 mM Tris, 1.5 mM EDTA, 10 mM monothioglycerol, 10 mM sodium molybdate, pH 7.4). The final concentrations of tracer were 5 nM [$^3$H]R2858 or 10 nM [$^3$H]R5020. Measurements were made in duplicate in the presence or absence of a 200-fold molar excess of the appropriate unlabeled steroid. Incubations were for 5 h at 23°C for estrogen binding and 4 h at 0–4°C for progestin binding; these time periods have been shown by time-course studies to allow attainment of equilibrium or steady-state conditions and complete exchange of bound hormone in mouse tissues [15–19]. A 25-fold molar excess of dexamethasone was added to the [$^3$H]R5020 solution to saturate glucocorticoid receptors. Under these assay conditions, binding of dexamethasone to progestin-binding sites is negligible. Following the incubation period, unbound steroid was removed from solution by adsorption to dextran-coated charcoal. Tubes were incubated for 10 min at 0–4°C with 0.3 ml 0.5% (w/v) Norit A and 0.05% (w/v) Dextran T-70 in buffer B and then centrifuged at 2000 $g$ for 10 min at 4°C. Aliquots of the supernatant (0.3 ml) were removed, added to 8 ml Aquasol and counted for radioactivity in a Beckman liquid scintillation counter (44% efficiency). Specific binding was obtained by subtracting nonspecific from total bound radioactivity and was expressed as pmol per mg DNA.

*Nuclear estrogen binding assay*

The procedure for this assay was adapted from methods described by Garola and McGuire[20]. Briefly, 0.2 ml nuclear extract was adsorbed onto 0.25 ml hydroxylapatite (HAP) slurry in buffer C (50 mM Tris, 10 mM $KH_2PO_4$, pH 7.4) for 30 min and then centrifuged at 1500 $g$ for 2 min. The resulting pellet was incubated with 0.1 ml 10 nM [$^3$H]R2858 in the presence or absence of a 300-fold molar excess of unlabeled R2858 for 3 h at 30°C. The HAP was then washed 4 times in buffer C. Hormone was extracted by overnight treatment of the HAP with 1 ml ethanol at 23°C. The HAP was then incubated for 1 h at 23°C with another 1 ml ethanol and the two ethanol supernatants were added to 7 ml Aquasol and assayed for radioactivity using a Beckman liquid scintillation counter (counting efficiency 44%).

*Statistical analysis*

To rule out seasonal differences, interassay variability, and other unknown influences on the data, the groups which we compared were neonatally treated in parallel fashion and the resultant tissues were assayed simultaneously. Student's $t$-test was used to analyze differences between control and DES-treated mice.

## RESULTS

*Mice treated with $10^{-1}$ $\mu$g DES/day*

Mice treated with $10^{-1}$ $\mu$g DES/day when compared with controls (Fig. 1) showed significantly lower levels of estrogen binding in both the vaginal FMW ($P = 0.026$) and the vaginal epithelium ($P = 0.013$). Within the FMW, nuclear estrogen binding was elevated in the treated group when compared to controls ($P = 0.037$). In the epithelium, nuclear estrogen binding tended to be lower in the

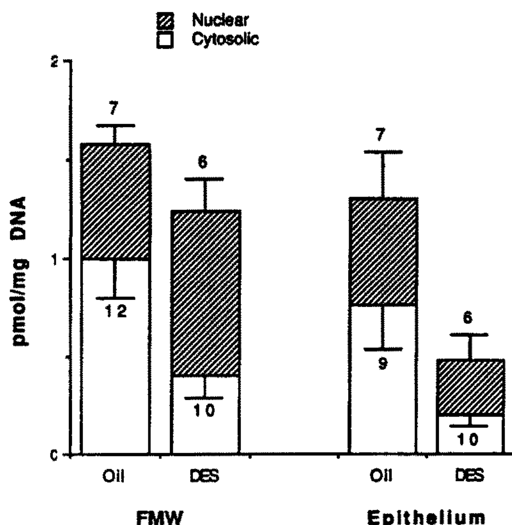


Fig. 1. Compartmentalization of vaginal estrogen binding in control mice (oil), and in mice neonatally injected with $10^{-1}$ $\mu g$ DES daily for 5 days. Vertical lines are SEM; $n$ values are above or below bars. FMW = fibromuscular wall.

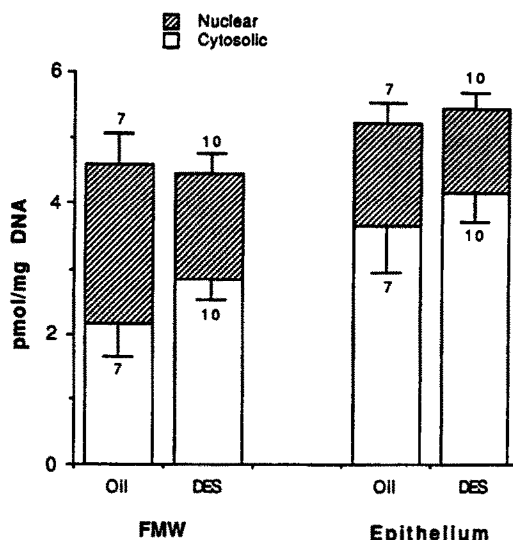


Fig. 3. Compartmentalization of vaginal estrogen binding in control mice (oil), and in mice neonatally injected with $10^{-3}$ $\mu g$ DES daily for 5 days. Vertical lines are SEM; $n$ values are above or below bars. FMW = fibromuscular wall.

treated animals although this difference was not statistically significant. The total estrogen binding is apparently decreased in the epithelium of the DES-treated animals. In the FMW the decrease in cytosolic binding is offset by the increase in nuclear binding, and it is unclear whether estrogen binding in the whole tissue decreases significantly. A feature of these neonatally induced changes is a shift in the predominant extent of estrogen binding from the cytosol to the nucleus. In fact, the nuclear:cytosolic ratio is approximately 4-fold greater in the neonatally DES-treated group relative to controls in both the FMW and epithelium.

In contrast to the decrease in cytosolic estrogen binding caused by neonatal DES treatment, the level of progestin binding increased in the DES-treated mice relative to controls (Fig. 2). These striking increases were statistically significant in both the epithelial ($P = 0.001$) and FMW ($P = 0.002$) compartments (Fig. 2). Nuclear progestin binding was not assayed since a reliable assay was not available.

### *Mice treated with $10^{-3}$ $\mu g$ DES/day*

Mice treated with $10^{-3}$ $\mu g$ DES/day when compared with controls (Fig. 3) did not show significantly different levels of vaginal cytosolic or nuclear estrogen binding in either the FMW or the epithelium.

The progestin binding in these DES-treated mice was significantly increased in the FMW ($P = 0.001$), but not in the epithelium (Fig. 4).

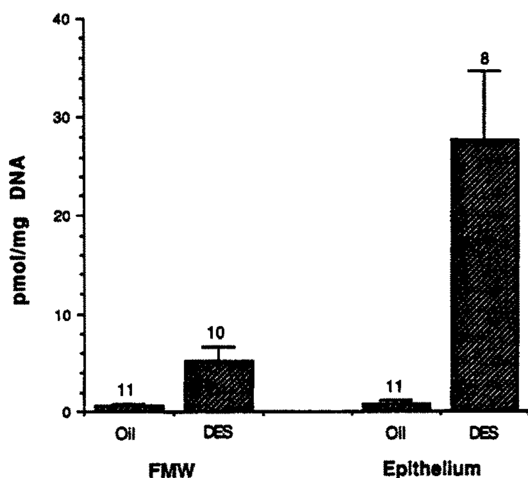


Fig. 2. Compartmentalization of vaginal cytosolic progestin binding in control mice (oil), and in mice neonatally injected with $10^{-1}$ $\mu g$ DES daily for 5 days. Vertical lines are SEM; $n$ values are above bars. FMW = fibromuscular wall.

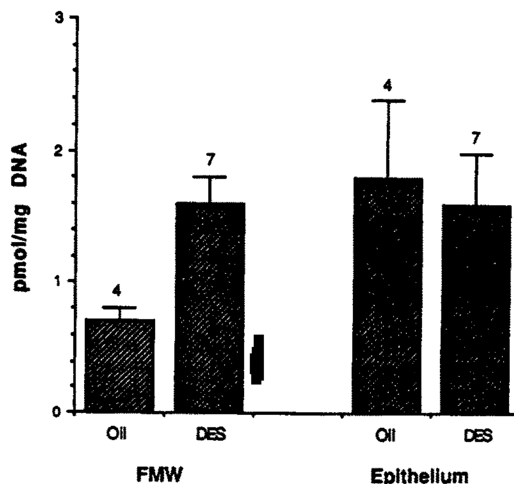


Fig. 4. Compartmentalization of cytosolic progestin binding in control mice (oil), and in mice neonatally injected with $10^{-3}$ $\mu g$ DES daily for 5 days. Vertical lines are SEM; $n$ values are above bars. FMW = fibromuscular wall.

## DISCUSSION

In both mouse [13] and human [21], estrogen receptors exist in both the epithelial and FMW compartments. The data presented here confirm this. However, in the present study, lack of tissue needed to conduct saturation analyses prevents any conclusion as to whether results seen are due to changes in the number or affinity of binding sites (or of both). Our data show that the lowered cytosolic estrogen binding of the adult mouse vagina following neonatal DES treatment [12] is a consequence of decreases in estrogen binding in both the epithelium and the FMW; and, since the FMW is approximately 4/5 of the vagina by DNA content [13], the FMW is therefore responsible for the majority of this decrease.

Changes in nuclear estrogen binding were tissue-dependent. In the epithelium, although not statistically significant, the trend was towards a decreased nuclear binding, i.e in the same direction as the cytosolic binding. In contrast, within the FMW, the neonatally treated adult tissue had increased nuclear binding.

Combining the adult FMW and epithelial estrogen binding data reveals two trends in the neonatally DES-treated group. First, total binding appears to be slightly lower in the FMW and significantly lower in the epithelium relative to control animals. Second, in both the FMW and epithelium, the nuclear:cytosolic binding ratio is approximately 4-fold greater in the neonatally DES-treated group. We believe this change represents a developmentally-induced difference between the treated and control animals. DES, neonatally injected, is no longer present in these 39-day-old mice, and the decreased cytosolic estrogen binding caused by neonatal DES treatment has been shown to persist for at least 12 months [12]. Recently Weisenberg *et al.*[22] reported similar data from studies comparing receptor levels in 2-month-old rat pituitary tissue and pituitary tumors induced by neonatal estrogen. Tumor tissue, relative to normal pituitary, had decreased cytosolic estrogen receptor levels and increased nuclear receptor levels. They proposed that the preferred nuclear localization of the receptors in the DES-treated animals may be due to nuclear changes in a rapidly dividing tissue, where mRNA and DNA synthesis would be expected to be much higher than normal; or it may reflect increased estrogen binding resulting in a change in receptor conformation and hence affinity to nuclear sites.

The data presented here suggest that estimates of tissue estrogen receptor content based on cytosolic assays alone can be misleading. Furthermore, these data could explain the discrepancies found in adult estrogen responsiveness following neonatal DES treatment. For example, acute estrogen-induced responses in the adult causally linked to the vaginal epithelial level of either total or cytosolic estrogen binding would be decreased in DES-treated animals relative to controls. In contrast, other acute estrogen-induced responses, either dependent on the level of nuclear binding in the FMW, or the nuclear:cytoplasmic binding ratio in either tissue, would be enhanced in treated animals.

Recent data [23–25] localize estrogen receptors within the nucleus and suggest that biochemical evidence of cytosolic receptors are homogenization artifacts. We suggest that the measured nuclear:cytoplasmic ratio of estrogen binding in these experiments reflects a relationship between the number and state of estrogen receptors and the number and affinity of nuclear binding sites for these receptors. It is also possible that intracellular modulators such as the proposed ligand binding protein [26] could alter the localization of steroid receptors.

No changes were observed in the levels of estrogen binding following the lower neonatal dose of DES. However, this dose of DES did cause an increase in progestin binding in the vaginal FMW. This suggests that a more sensitive biochemical assay to detect DES-induced developmental changes would be based on progestin binding and not estrogen binding. In addition, the site of this change, the FMW, underscores the importance of the stroma in development [27].

The large increases in progestin binding following the higher neonatal dose of DES could prove useful in examining a "spare steroid receptor" hypothesis. DES-treated animals may have more vaginal progestin receptors than are necessary to elicit a maximal progestin response. If so, these receptors could act in an inhibitory fashion by competing for ligand with a more "effective" population of DNA-linked receptors.

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