Estradiol Down-Regulation of the Rat Uterine Estrogen Receptor (43191)

KEVIN L. MEDLOCK,*[,1] C. RICHARD LYTTLE,[†] NICKY KELEPOURIS,[†,2] ERIN D. NEWMAN,[3] AND DANIEL M. SHEEHAN*[,‡]

Division of Reproductive and Developmental Toxicology, Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas 72079; Department of Obstetrics and Gynecology,[†] Division of Reproductive Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and Departments of Biochemistry and Pharmacology and Interdisciplinary Toxicology,[‡] University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205*

Abstract. **We have previously shown that neonatal exposure of rats to pharmacologic doses of diethylstilbestrol via daily injections resulted in a significant decrease in the estrogen-binding capacity of the uterine estrogen receptor (ER). In this study, we examined the effects of physiologic and pharmacologic doses of estradiol (E_2) administered to adult ovariectomized rats via Silastic implants. Two days after implantation, uteri were removed, weighed, and homogenized, and ER levels were determined in the supernatant (hydroxylapatite assay) and low-speed pellet (nuclear exchange assay). Implants containing E_2 concentrations of 0.005 or 0.05 mg/ml increased cytosolic but not total ER-binding capacity, whereas 0.5 or 5.0 mg of E_2/ml implants decreased the binding capacity of cytosol ER to 40% and total ER to 50% of control values. The 0.005-mg/ml dose increased cytosol ER without increasing uterine weight; all higher doses significantly increased uterine weight. Determination of ER protein by an ER radioimmunoassay showed the same extent of reduction of ER concentration as the binding assays, demonstrating that the loss in E_2 binding capacity is homologous down-regulation. The down-regulation of ER was maximal at 24 hr and was completely reversible after implant removal, although the time required to recover from down-regulation was dose dependent. Uterine weight also returned to control levels slowly after implant removal. Neither the sedimentation rate of the down-regulated ER nor the K_d of the cytosolic ER changed following long-term implantation; however, the K_d of the nuclear ER decreased significantly. This is the first demonstration of *in vivo* homologous down-regulation of uterine ER. ER down-regulation may play a role in several biologic processes.** [P.S.E.B.M. 1991, Vol 196]

A variety of toxicologic effects have been seen in animals and humans following the administration of pharmacologic doses of estrogens (1-4). Estrogen toxicity appears to be mediated by the estrogen receptor (ER) (5). Numerous investigators have examined the estrogen regulation of ER concentration in various target tissues. Several hours following exposure to estrogen, the total ER ("cytosolic" plus "nuclear") content is decreased (6, 7). Subsequently, ER levels increase such that by 24 hr after injection, the total ER concentration recovers to and may exceed the pretreatment values (8). Whereas the loss of uterine ER has been explained as receptor "processing" (9), it is unclear if ER disappears or if ER protein is conserved but is unable to bind ligand (9-11). By contrast, glucocorticoids are known to induce receptor loss, an example of homologous down-regulation (12, 13). Studies of ER regulation are important not only because the ER is one of a group of proteins regulated by both estrogens and progesterone (8, 14) but also because target tissue responsiveness to estrogens is regulated by ER (15). We earlier reported that diethylstilbestrol (DES) given daily to newborn rats at pharmacologic doses (equivalent to the doses women received for threatened pregnancies)

[1] To whom requests for reprints should be addressed.

[2] Present address: Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104.

[3] Present address: Department of Physiology, University of California, San Francisco, CA 94143.

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resulted in a significant decrease in total uterine ER concentration after 5 days of treatment (5). Estrogen injections provide a rapid increase and then decrease in serum estradiol (E_2) levels, a pattern unlike that seen in normal reproductive cycles. Furthermore, it is experimentally easier to work with adult rather than neonatal rats and to implant a single capsule releasing E_2 than to inject animals daily. For these reasons, we investigated estrogen regulation of ER concentration in adult rats exposed to constant levels via release from Silastic capsules.

This report extends to adults our observations on the estrogen regulation of ER in neonates and demonstrates that the ER loss is dose dependent, reversible, and accompanied by a loss of receptor protein. Finally, the experimental ease with which varying uterine ER concentrations can be achieved and maintained appears useful in further studies of ER regulation.

Materials and Methods

DES and 17β-E_2 were obtained from Research Plus Steroid Laboratories, Inc. (Denville, NJ) and [^{3}H]17β-E_2 (sp act, 90–114 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals used were laboratory grade.

Female Sprague-Dawley CD rats raised on site at the National Center for Toxicological Research (Jefferson, AR) were ovariectomized at 2 to 3 months of age. One week later, groups of rats were implanted for varying lengths of time with Silastic capsules while untreated ovariectomized groups served as controls. The implants contained E_2 suspended in sesame oil at concentrations of 0.0005, 0.005, 0.05, 0.5, and 5.0 mg/ml (absolute amount of E_2 in implant = 0.005, 0.05, 0.5, 5.0, and 50.0 μg, respectively) and were inserted subcutaneously in a small mid-dorsal incision that was closed with a wound clip (2). All ovariectomies, implantations, removal of implants, and sacrifices (by cervical dislocation) were done under light ether anesthesia. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

After killing, animals were weighed individually and the uteri were removed, stripped of adhering mesentery, weighed, and homogenized in 0.01 *M* Tris, 0.0015 *M* EDTA, and 0.001 *M* dithiothreitol (TED) buffer (pH 7.4) at a concentration of 30 mg of tissue/ml. Nuclear ER levels and dissociation constants were determined by the nuclear exchange assay described previously (16) with the addition of reducing agent to avoid interference by type II estrogen-binding sites (17). The supernatant from the 800*g* centrifugation of the original homogenate was further centrifuged at 30,000*g* for 1 hr at 0°C and ER concentration determined by hydroxylapatite assay (18). For convenience, this will be designated as cytosolic ER. The concentrations of [^{3}H]E_2 in both the nuclear and cytosolic assays ranged from 0.25 to 10.0 n*M*; tubes containing a 100-fold *M* excess of DES were used for determination of nonspecific binding. Samples were counted in a TM Analytic III liquid scintillation counter (Searle Analytic, Des Plaines, IL).

In other experiments, uterine ER was extracted by homogenizing uteri (30 mg/ml) in TED containing 0.4 *M* KCl. The homogenate, incubated at 0–4°C for 1 hr, was then centrifuged at 30,000*g* for 1 hr. The high salt extracts were centrifuged on 5–20% sucrose gradients in 0.4 *M* KCl-TED for determination of ER sedimentation properties. The antibody determinations of estrogen receptors were performed using the Abbott ER-enzyme immunoassay method (19) with minor modification. Rat uteri were snap-frozen and stored at −80°C until assayed for receptor. Frozen tissue were homogenized in a 10 m*M* Tris-HCl pH buffer containing 1.5 m*M* EDTA, 5.0 m*M* sodium molybdate, 1 m*M* monothioglycerol, and 0.4 *M* KCl using a Polytron fitted with an ST-10 generator. All procedures were carried out at 4°C. The homogenate was centrifuged at 38,000*g* for 40 min and the resulting supernatant was used in the ER-enzyme immunoassay. Protein concentration of the supernatant was determined using the Bradford Assay with bovine serum albumin as standard (20).

Statistical analyses were conducted on all data by a one-way analysis of variance followed by Duncan's multiple range test with a significance level of $P \leq 0.05$.

Results

Estrogen Receptor Concentration at Various E_2 Doses. Two days after implanting Silastic capsules, the concentration of the cytosolic ER was increased significantly above controls at implant concentrations of 0.005 and 0.05 mg/ml E_2 (Fig. 1A). With 0.5 and 5.0 mg/ml E_2, the cytosolic ER concentration decreased significantly to approximately one-third that of controls. The nuclear ER concentration was not significantly decreased at any of the doses. The total ER concentrations followed the same trend as the cytosolic ER concentrations, although there was no significant difference between ER levels in the controls and those implanted with the three lowest E_2 concentrations. The uteri of rats exposed to the two lowest E_2 concentrations did not show a significant increase in weight (Fig. 1B). At concentrations of 0.05 mg of E_2/ml or higher, uterine weight increased over 2-fold (Fig. 1B). Typical Scatchard plots for nuclear and cytosolic ER binding are shown in Figure 2 for control and 5.0 mg/ml E_2-treated animals. No effect of estrogen treatment was noted on the shape of the curves, although there was a significant decrease in the nuclear K_d at concentrations of 0.05 mg of E_2/ml and higher (Table IA).

Estrogen Receptor Suppression with Time. To determine the extent of ER suppression at various times

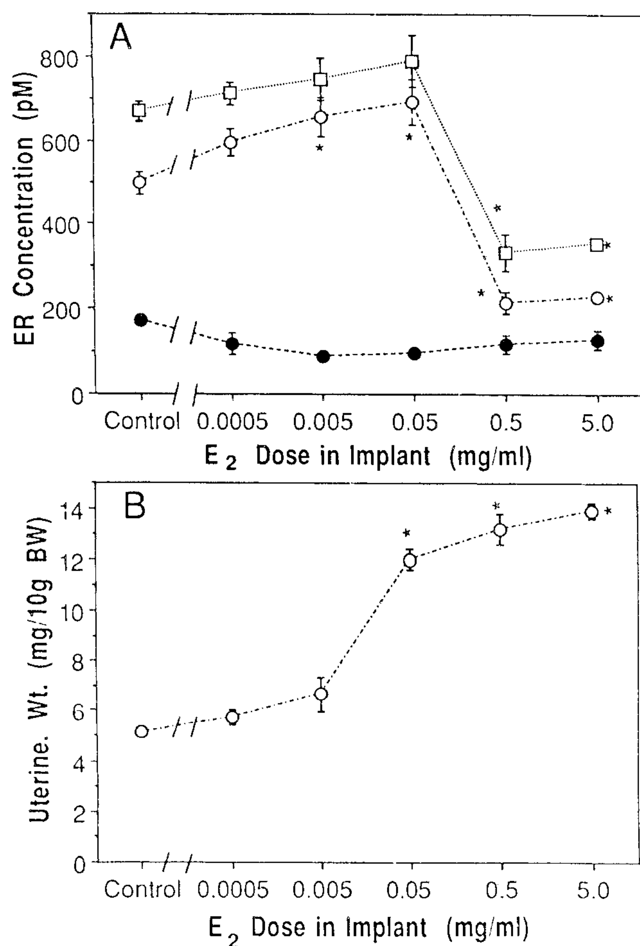

Figure 1. Dose-response curves for (A) cytosolic (○), nuclear (●), and total (□) ER concentrations and (B) uterine weight following 2 days of implantation with various concentrations of E_2. The data are expressed as the $\bar{X} \pm$ SE with an n of at least three from independent experiments. $^*P \leq 0.05$ compared with controls.

after E_2 implantation, the 5.0 mg of E_2/ml implant was used to ensure maximal ER suppression. Following implantation, there was a significant decrease in the cytosolic ER level for all days examined with the exception of Day 10 (Fig. 3A). This point, while significantly increased over the Day 1 value, does not appear biologically significant, since it is preceded and followed by significantly decreased values. The average decrease over 14 days was 50%. The length of time of implantation had no effect on the nuclear ER concentration. The total ER concentration showed a significant decline of more than 50% by Day 1 and remained essentially unchanged through Day 14 (Fig. 3A). The time course of uterine weight gain showed that uterine weight increased significantly through Day 7, after which it plateaued at about three times that of the controls (Fig. 3B).

Estrogen Receptor Recovery from Suppression. To determine if the E_2 effect on ER suppression was reversible, 5.0 mg of E_2/ml implants were removed after 1 or 7 days, and ER levels were determined at

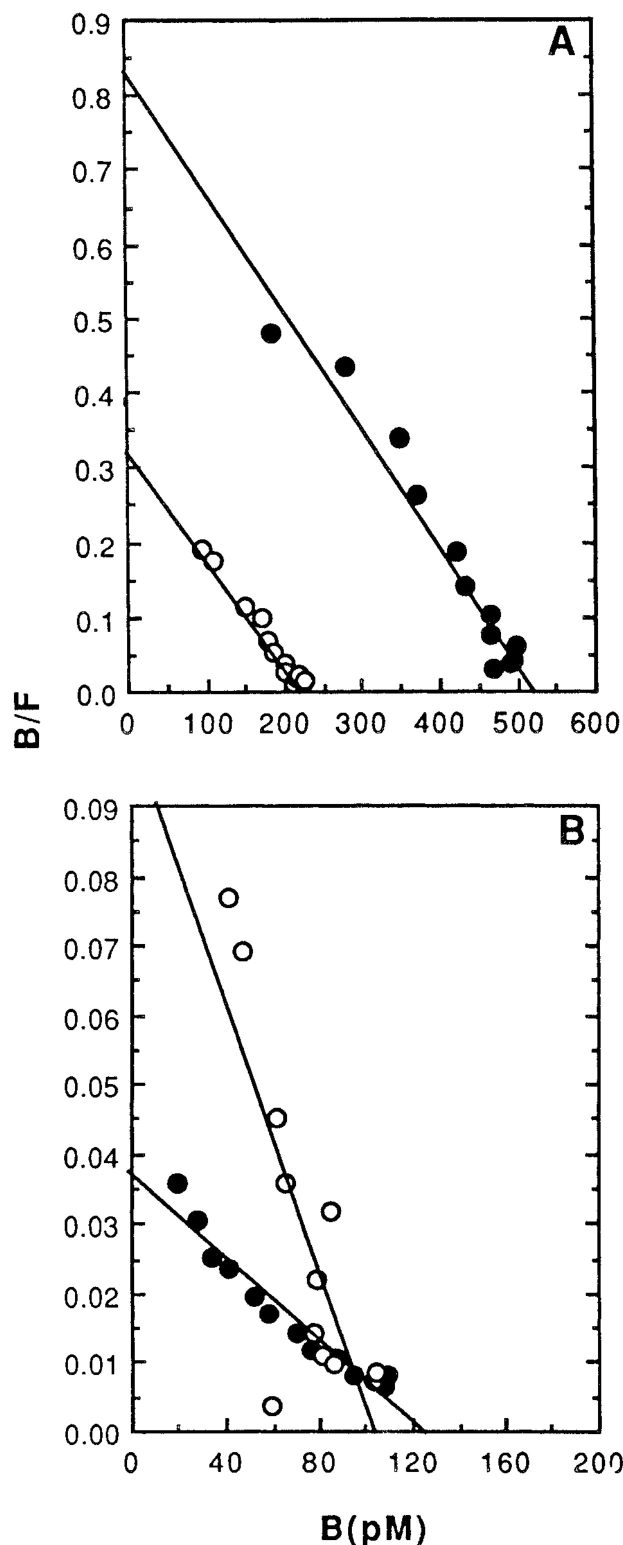

Figure 2. Representative Scatchard plots of cytosolic (A) and nuclear (B) ER binding of E_2 from control rats (●) and rats implanted with 5.0 mg of E_2/ml for 2 days (○). The K_d (in nM) are (A) controls, 0.62 and E_2 treated, 0.69 and (B) controls, 3.35 and E_2 treated, 0.86.

Table I. Effect of Implant Dose, Exposure Time, and Removal Time on K_d of Cytosolic and Nuclear Binding Sites[a]

Treatment groups	n	Nuclear	Cytosolic
A. Dose			
Control[b]	74	3.39 ± 0.22	0.80 ± 0.07
0.0005 mg of E_2/ml	13	2.41 ± 0.24	0.88 ± 0.12
0.005 mg of E_2/ml	4	2.45 ± 0.71	1.28 ± 0.28
0.05 mg of E_2/ml	11	0.91 ± 0.08[c]	0.76 ± 0.14
0.5 mg of E_2/ml	9	0.92 ± 0.10[c]	1.06 ± 0.30
5.0 mg of E_2/ml	5	0.92 ± 0.06[c]	0.71 ± 0.02
B. Length of exposure			
Implanted 1 day	11	0.87 ± 0.11[c]	1.23 ± 0.21
Implanted 2 days	5	0.90 ± 0.06[c]	0.71 ± 0.02
Implanted 7 days	6	0.98 ± 0.14[c]	0.70 ± 0.07
Implanted 10 days	2	0.60 ± 0.02[c]	1.40 ± 0.24[c]
Implanted 14 days	8	0.50 ± 0.08[c]	0.80 ± 0.21
C. Recovery from exposure			
Implanted 1 day, removed 1 day	11	1.67 ± 0.22[c]	0.74 ± 0.07
Implanted 7 days, removed 1 day	5	4.15 ± 0.71	1.19 ± 0.35
Implanted 7 days, removed 3 days	3	2.15 ± 0.57[c]	0.77 ± 0.13
Implanted 7 days, removed 7 days	9	2.21 ± 0.28[c]	0.59 ± 0.03[c]

[a] Values are expressed as $\bar{X} \pm$ SE.
[b] Means of control K_d were derived from control values of all regimens and thus are appropriate for all treatment groups.
[c] Indicated a significant difference ($P \leq 0.05$) from controls.

various times thereafter. One day after being inserted, the implant was removed. One day later, both the cytosolic and total ER concentrations had recovered to control levels (Fig. 4A), although the nuclear ER concentrations remained significantly lower than controls. The recovery to control values in animals that had been implanted for 7 days was much slower. One day after removal, there was still a significant decrease in the nuclear ER level. The total ER level did not show a significant change from the 7-day implant level. Three days after implant removal, the values for cytosolic and total ER concentrations had recovered to control levels, while nuclear ER level remained below the control value. Seven days after implant removal, the cytosol and total ER concentrations were significantly higher than in controls, although the nuclear concentration was still significantly lower than in controls.

Uterine weights after the implants were present for 1 day increased 2-fold over those of controls (Figs. 3B and 4B) and remained elevated after the implants had been removed for 1 day (Fig. 4B). After the implants had been present for 7 days, the uterine weights were three times the control values (Figs. 3B and 4B). Following removal of the implants, 7 days were required for uterine weights to return to control values (Fig. 4B).

Suppression and Recovery Effects on K_d. There were no consistent significant effects of E_2 dose, length of exposure, or length of recovery on cytosolic ER K_d (Table I). However, the nuclear K_d decreased in 12 of 13 treatment conditions in which uterine weight was significantly elevated. There was no change in nuclear K_d in the two E_2 treatment conditions that did not alter uterine weight. Figure 2D shows a Scatchard plot for nuclear ER with a lowered K_d.

Sedimentation Properties of ER. Sucrose gradient analysis of ER extracted by KCl showed no difference between control and implanted animals with respect to sedimentation profile (Fig. 5). The gradients also demonstrate the decline in ER concentration following E_2 implant.

Immunoassay of ER. The uterine ER protein levels detected by the immunoassay were about twice the total ER determined by combining the nuclear and cytosol ER levels (Table II). Both binding site and ER protein concentrations were equally reduced following 2 days of E_2 implantation.

Discussion

Estradiol suppression of uterine ER-binding capacity is dose dependent, reversible, and accompanied by a corresponding loss of immunoreactive ER. The latter point distinguishes down-regulation from an alteration to an ER form that fails to bind E_2 (21). Homologous down-regulation of hormone receptors by their ligands has been reported for both peptide and other steroid hormones (12, 13, 22, 23). Although this is the first report of estrogen down-regulation of uterine ER, estrogens have been shown to down-regulate ER in estrogen-responsive cells (7, 24). Both ER (7, 24) and ER mRNA (25) decline in E_2-treated MCF-7 cells to about 50% of control values. Our results from *in vivo* treatment show a similar extent of suppression.

It is significant that high levels of E_2 are required for down-regulation to occur (implant E_2 concentration

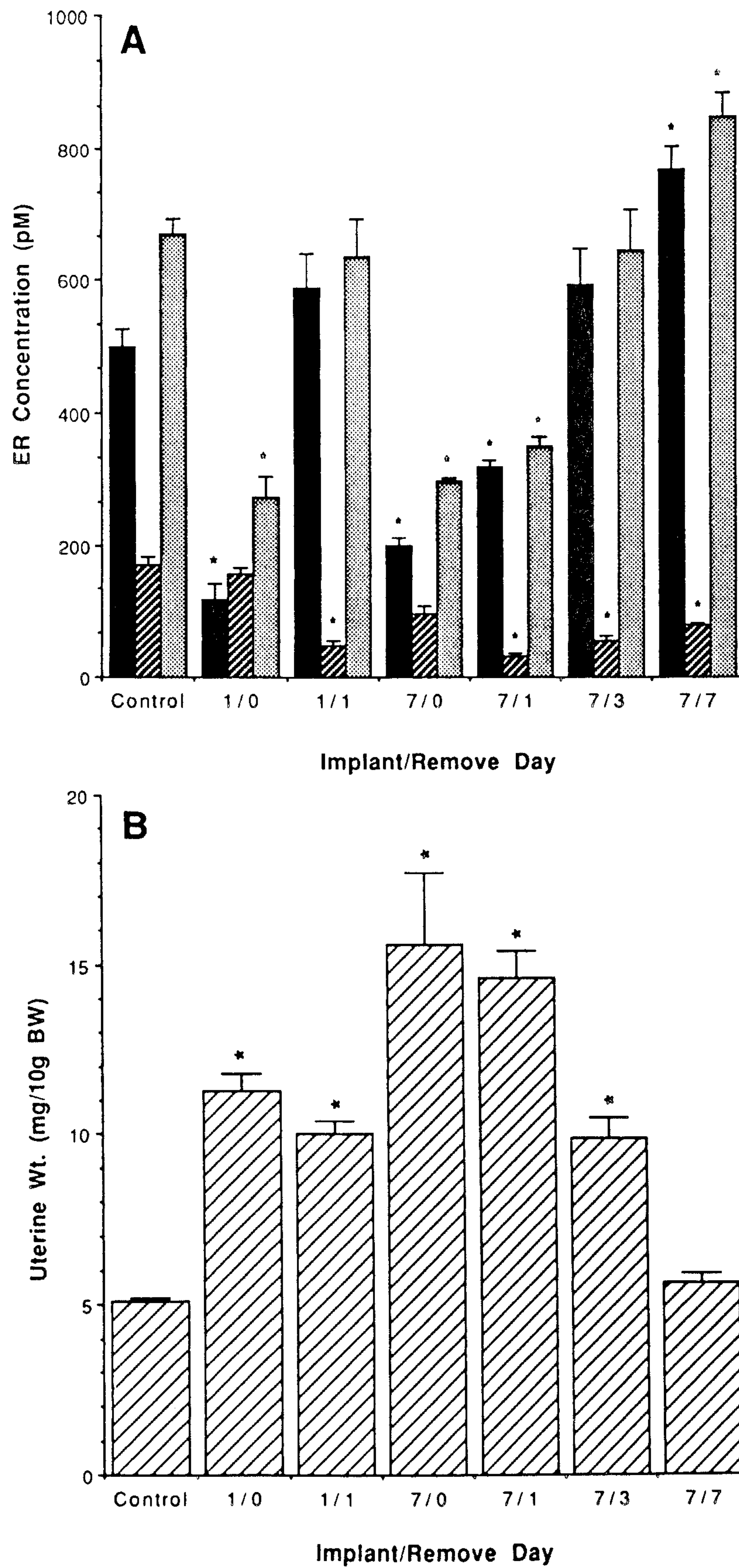

Figure 4. Effects of different implantation (5.0 mg of E_2/ml) removal times on (A) cytosolic (solid bars), nuclear (dark hatched bars), and total (light hatched bars) ER concentrations and (B) uterine weight. The first number of each set represents the number of days the implant was in the animal before it was removed; the second number represents the number of days between removal and sacrifice. The data are expressed as the $\overline{X} \pm$ SE with an n of at least three from independent experiments. $^*P \pm 0.05$ compared with controls.

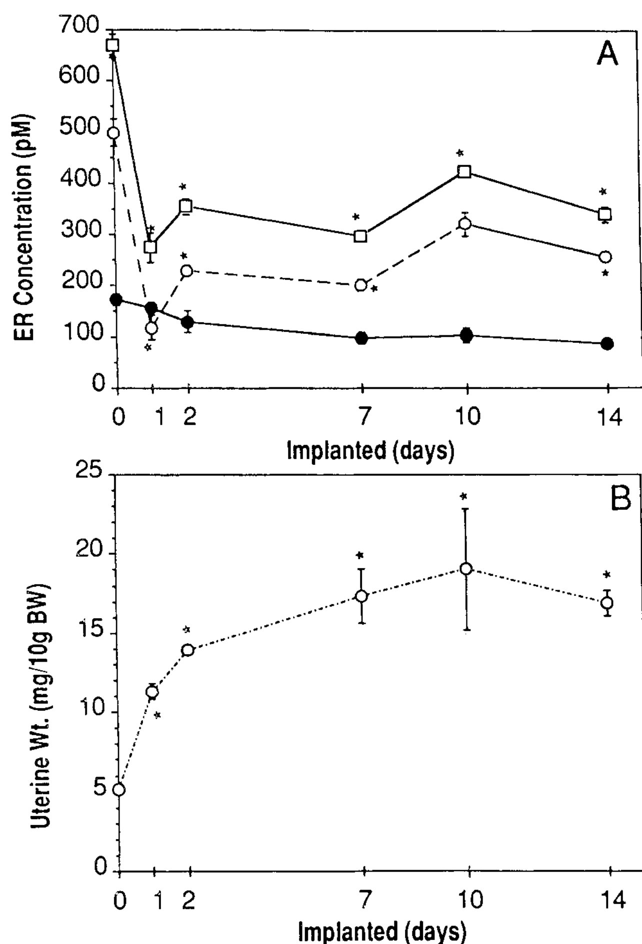

Figure 3. Effect of length of implantation time with 5.0-mg/ml E_2 on (A) cytosolic (○), nuclear (●), and total (□) ER concentrations and (B) uterine weight. The data are expressed as the $\bar{X} \pm$ SE with an *n* of at least three from independent experiments. $^*P \leq 0.05$ compared with controls.

≥ 0.5 mg/ml). We have previously shown that the 5.0-mg/ml implant produced a steady serum E_2 level of 200 pg/ml 2–14 days after implantation (2). Lower implant concentrations of E_2 in the physiologic range (as shown by the uterine weight increase) do not down-regulate ER. In fact, there is a significant increase in the cytosolic ER concentration at the E_2 concentration of 0.005 mg E_2/ml that is similar in magnitude to the increase in ER found in immature rats 24 hr after a single estrogen injection (18, 26). The homologous regulation (both up- and down-regulation) of ER appears to be a cytosolic phenomenon. This is not unexpected, since the reciprocal fluctuations seen in nuclear and cytosolic ER concentrations are short-term events occurring within the first 24 hr following the injection of an estrogen (18). With continuous dosing, once these initial events have occurred, no further changes are seen. There does appear to be a dynamic equilibrium in ER synthesis and loss as evidenced by the loss of nuclear receptor following implant removal after either 1 or 7 days of implantation. Physiologically, the loss can be related to simultaneous loss in uterine weight. While an increase in ER concentration 24 hr following E_2 administration is normally associated with a uterine weight increase, our results and those of Diaz-Chico *et al.* (27), demonstrate that ER regulation by E_2 can occur at doses that fail to induce uterine growth. Jakesz *et al.* (28) found no loss of ER during 6 hr of continuous exposure to physiologic low E_2 levels but did not examine higher E_2 doses.

The suppression of ER is not associated with a change in the K_d of the cytosolic ER or in the sedimentation rate of the high salt extracted receptor. However, there is a significant increase in the nuclear ER affinity for E_2 after E_2 treatment that is strongly associated with elevated uterine weight. The increase in nuclear ER affinity and the suppression of both cytosolic and total ER are reversible since implant removal results in all parameters returning to control values. Changes in ER affinity for E_2, which have been described in calf uterus (29) and chick oviduct (21), appear to be dependent on the state of ER phosphorylation. Although ER down-regulation is maximal within 24 hr after implantation, the time required to recover from suppression is dependent on the length of time the implants remained in place. Since uterine weight also recovered more slowly after longer implant times, serum E_2 levels may be declining more slowly than after short exposures, perhaps due to a body fat depot. However, following neonatal exposure to high levels of DES, only a partial recovery of ER levels is seen, and the time course of recovery is longer than can be accounted for by storage in fat depots (5) (unpublished observations). Whereas the ER down-regulation phenomenon can be seen following continuous E_2 dosing in both the neonate and the ovariectomized adult, the interpretation of neonatal results is more complicated. Neonatal treatment with estrogens causes a permanent alteration in the hypothalamo-hypophyseal-ovarian axis resulting in a failure of ovarian cyclicity (30). In the ovariectomized adult, developmental changes in hypothalamic functions are not a confounding variable.

Both the exchange assay of non-KCl-extracted ER and the antibody assays of the KCl-extracted ER show suppressed ER levels following continuous E_2 exposure. The total ER level measured by the exchange assay is half that measured by immunoassay in both control and E_2-treated rats. This difference in results between the binding assay and immunoassay has been reported previously (19, 31) and is consistent over a wide range of ER concentrations. More important than the agreement of ER levels between the two assays is the finding of an identical extent of suppression in both assays, which demonstrates that ER suppression is a consequence of a lowered ER protein concentration.

Progesterone not only down-regulates its receptor (32) but also lowers the level of nuclear ER when given

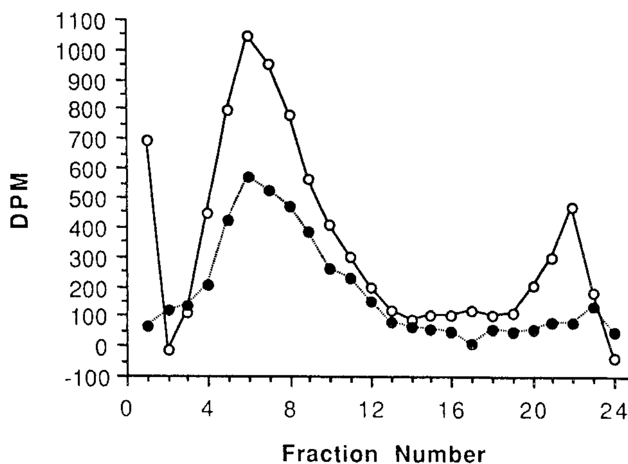

Figure 5. Representative sucrose gradients of KCl-extracted ER in uteri from control rats (O) and rats implanted for 2 days with 5.0 mg of E_2/ml (●).

Table II. Comparison of Exchange Assay with Monoclonal Antibody Assay for ER Determination[a]

	Control	High-dose E_2	% Suppression
Exchange assay	669 ± 24	355 ± 15	47
Antibody assay	1179 ± 137	568 ± 82	52

[a] Values are $\bar{X}$ ± SE (*n* of at least three independent experiments), presented as p*M* E_2 bound.

in the presence or absence of E_2 (14, 33, 34). Progesterone is thought to down-regulate uterine ER in the second half of the reproductive cycle (35), but our findings suggest that the high serum concentration of E_2 at proestrus may also contribute to ER down-regulation. Progesterone is not involved in the down-regulation we observe, since the rats are ovariectomized before implantation and thus should have negligible levels of progesterone.

The uteri of rats implanted with 5.0 mg of E_2/ml for at least 2 days do not continue to grow (2) (Fig. 3B). Since uterine refractoriness to continued E_2 exposure occurs under conditions causing ER down-regulation, it is possible that ER down-regulation causes or is partially responsible for the refractory condition. Consistent with this suggestion are the findings of Stormshak *et al.* (36), who demonstrated that ER levels, DNA and protein synthesis, and glucose oxidation in uteri of rats injected daily with 1.0 μg of E_2 peaked at 1 day and declined to control levels by 3 days. Down-regulation of ER may also be important in high-dose estrogen treatment, which has been shown to induce regression of ovarian-dependent breast cancers (37).

Estrogens are well-known toxicants, especially when given chronically and without concurrent cyclical progesterone treatment. The use of new, low-level chronic estrogenization regimens (dermal patches, creams, implants, and depot formulations) would not be expected to down-regulate ER based on our results. However, our data do demonstrate that dose, route, and duration of treatment may be important in estrogen regulation of ER levels.

The data presented in this article demonstrate that under certain conditions, E_2 is an homologous down-regulator of ER. Since these conditions are found in the human and rodent reproductive cycle and can cause the uterine refractory state, breast tumor regression, and toxic responses, ER down-regulation by estrogens may be an important phenomenon. The experimental ease with which ER concentrations can be maintained is an important finding of this study and appears useful for further studies of *in vivo* E_2 regulation of ER.

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