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Chronic Estrogen-Induced Alterations in Adrenocorticotropin and Corticosterone Secretion, and Glucocorticoid Receptor-Mediated Functions in Female Rats*

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ABSTRACT

The effect of estrogen (E) on the hypothalamic-pituitary-adrenal axis was investigated in female Sprague-Dawley rats. Animals were bilaterally ovariectomized (OVX), and a Silastic capsule (0.5 cm) containing 17β-estradiol was sc implanted. Control animals received a blank capsule. Animals were killed 21 days later. In E-treated rats, we found significantly higher corticosterone (CORT) peak levels 20 min after a 5-sec footshock (1.0 mamp) or exposure to ether vapors ($P < 0.05$) compared to those in OVX controls. In addition, the recovery of the ACTH and CORT responses to footshock stress was significantly prolonged ($P < 0.05$) in the presence of E. Furthermore, the ACTH and CORT secretory responses to ether stress could be suppressed by exogenous RU 28362 (a specific glucocorticoid receptor agonist; 40 μg/100 g BW for 4 days) in OVX controls ($P < 0.05$), but not in E-treated animals. These data suggest that E can impair glucocorticoid receptor-mediated delayed or slow negative feedback.

Consequently, we examined the influence of E on mineralocorticoid and glucocorticoid receptor concentrations using *in vitro* binding assays. E did not alter mineralocorticoid or glucocorticoid receptor concentrations in any of the brain regions examined. The administration of RU 28362 (40 μg/100 g BW for 4 days) to OVX control or E-treated rats significantly down-regulated hippocampal glucocorticoid receptor ($P < 0.02$) in control rats only. In contrast, aldosterone administration (40 μg/100 g BW for 4 days) significantly down-regulated hippocampal glucocorticoid receptor ($P < 0.0008$) in both control and E-treated animals. Thus, E treatment results in a loss of the glucocorticoid receptor's ability to autoregulate; this suggests that E may cause a functional impairment of the glucocorticoid receptor even though receptor binding appears normal.

These findings suggest that hyperactivation of the hypothalamic-pituitary-adrenal axis after stress in E-treated rats is due in part to impaired glucocorticoid receptor-mediated slow negative feedback. (*Endocrinology* **131:** 1261–1269, 1992)

ACTIVATION of the hypothalamic-pituitary-adrenal (HPA) axis is a characteristic physiological response to stress. Previous studies have demonstrated that the estrogen (E) status of the female rat may affect its endocrine response to stress. It has been shown that a sex difference exists in both circulating corticosterone (CORT) and the CORT response to stress, with females having higher levels than males (1, 2). Additional studies have shown that ovariectomy (OVX) reduces basal CORT levels, and E replacement increases them (3–5). This is consistent with studies showing that basal (5–8) and stress-responsive (5, 6, 8, 9) CORT levels are highest on proestrus when E levels are the highest. Finally, it has been shown that female rats receiving E replacement after OVX have significantly higher poststress CORT levels than controls (5, 8).

At present, the factors that underlie the apparent effect of E on CORT secretion have not been examined. Several possibilities exist, including E's effects on CRF synthesis or secretion, anterior pituitary gland (AP) sensitivity to CRF, or CORT negative feedback mechanisms. Previous studies have shown that E may directly effect levels of CRF immunoreactivity and mRNA (10–12). While chronic E treatment decreased hypothalamic CRF immunoreactivity (10), CRF mRNA was found to be elevated on the afternoon of proestrus, at the approximate time of the E-induced preovulatory surge of LH (12). Using hypothalamic extracts to stimulate AP release of ACTH, Coyne and Kitay (13) demonstrated decreased sensitivity of the AP after OVX, which was partially reversed by E. Studies in the aged rat showed that deficits in hippocampal CORT receptors are associated with the hyperactivation of the HPA axis found in response to stress (15, 16). This pattern is similar to the one we described for the E-treated animal (17).

Negative feedback regulation of the HPA axis, both fast and delayed, is mediated predominately through the binding of CORT to intracellular CORT receptors found in the hippocampus (HIPP), hypothalamus, and AP (18–20). Previous studies have classified receptors for CORT into type I and type II receptors (21 , 22), which have been shown to be the products of the mineralocorticoid receptor (MR) gene and the glucocorticoid receptor (GR) gene, respectively (23, 24). In the following studies, we will employ the designations of MR and GR for the type I and type II receptors. Both receptor types have been implicated in negative feedback by CORT (25, 26), and both are found in varying amounts at the three

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major sites involved in negative feedback.

In the following studies we 1) characterized the ACTH and CORT responses to stress in female rats in the absence of E (OVX) or with chronic E replacement, 2) examined the prospect that alterations in CORT-delayed negative feedback are contributing to differing hormonal responses in the presence and absence of E; and 3) explored the possibility that the E status of the animal may influence CORT receptor regulation.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (250–300 g; Sasco, Omaha, NE) were housed in environmentally controlled quarters, with a 12-h light, 12-h dark cycle (lights on at 0700 h) and food and water available *ad libitum*. Adrenalectomized (ADX) rats were maintained on 0.9% saline immediately after surgery.

Animals were bilaterally OVX under ether anesthesia, followed either by sc implantation of a 0.5-cm Silastic capsule (id, 0.2 in.; od, 0.37 in.; Dow-Corning, Midland, MI) filled with 17β-estradiol (E; Sigma Chemical Co., St. Louis, MO) or a sham operation (control). The average serum estrogen levels achieved with this E capsule treatment are approximately 75 pg/ml (27). Bilateral adrenalectomy (ADX) was performed under ether anesthesia by making two dorsal flank incisions. Three days before blood sampling, Silastic catheters (id, 0.078 in.; od, 0.125 in.; Dow-Corning) were inserted into the right atrium via the right jugular vein, exteriorized by passage under the skin to the back of the neck, and then connected to a vascular access port (Access Technologies, Skokie, IL) attached to the skin of the animal's back.

Experimental protocols

Exp 1a: effect of E on ACTH and CORT responses to footshock stress, a repeated sampling paradigm. The time course of the ACTH and CORT responses to stress was examined to determine if E treatment has a similar effect when given in adulthood as that previously reported when OVX and E treatment were administered prepuberally. We used indwelling right atrial cannulae for repeated sampling from individual animals during the recovery period after footshock stress. These studies are important in determining whether the observed effect of E is through a disruption of the negative feedback actions of CORT. Female rats were bilaterally OVX to reduce circulating E levels. Half of the animals then received an sc implantation of a 0.5-cm Silastic capsule filled with 17β-estradiol, while control rats were OVX and sham treated. Animals were handled for 3 min each day for 1 week before testing. Eighteen days after treatment, Silastic tubing (id, 0.078 in.; od, 0.125 in; Dow-Corning) was inserted into the right atrium via a nick in the right jugular vein and externalized by connecting the tubing to a vascular access port (Access Technologies), which was secured to the skin of the animal's back. Stress responses were tested 3 days later (day 21). On the day of testing, PE-50 tubing was connected to the ports 3 h before stress testing. The stressor used was 5 sec of inescapable footshock (1.0 mamp). Animals were placed into the shock chamber and returned to their homecage immediately after the shock was administered. Blood samples (0.5 ml) were taken 0, 5, 10, 15, 20, 30, 60, and 120 min after return to their homecage. An equal volume of rat red blood cell preparation was replaced after each sampling to maintain hematocrit and osmotic balance (28). Plasma was removed from samples and frozen at −70 C until assayed for ACTH and CORT by RIA.

Exp 1b: effect of E on the CORT response to footshock stress, single point measurements. Data from Exp 1a suggested that E treatment impaired negative feedback. To eliminate the effects of cannulation, we used animals killed at different time points to examine the pattern of the CORT response after footshock stress. OVX and E treatment were as described in Exp 1a. Animals were handled for 3 min each day for 1 week before testing. Twenty-one days after treatment, animals were removed from their homecages, subjected to 5 sec of inescapable footshock (1.0 mamp) in a shock chamber, and immediately returned to their homecages. Nonstressed controls were killed immediately upon removal from their homecage. Footshocked animals were killed 20, 45, or 70 min after the stress. Animals were killed by decapitation, trunk blood was collected, and plasma was removed and frozen at −70 C. Plasma samples were assayed for CORT levels by RIA.

Exp 2: effect of E on the CORT response to ether stress. This experiment was performed to determine whether 1 min of ether stress would prove a comparable stressor to the footshock used above. Ether stress would allow for collection of the 0 and 20 min poststress samples by tail vein bleeding from the same animal without the need for cannulation. Adult female rats were OVX and E treated as described in Exp 1. Twenty-one days after treatment, rats were stressed by brief exposure to ether vapor. The ether stress protocol consisted of placing animals in a jar containing ether-soaked gauze and removing them after 1 min. The 0 and 20 min poststress blood samples (0.5 ml) were subsequently collected from the tail veins. Plasma was removed and stored at −70 C until assayed for CORT by RIA.

Exp 3: effect of E on dexamethasone (DEX) and RU 28362-mediated suppression of the neuroendocrine stress response. Our previous studies (Exp 1 and 2) suggest that an impairment of the negative feedback mechanism of CORT on the HPA axis is present after chronic E treatment. In this study we employed the synthetic glucocorticoid DEX (9α-fluoro-16α-methylprednisolone; Sigma Chemical Co., St. Louis, MO) or the specific GR agonist RU 28362 (11β,17β-dihydroxy-6-methyl-17α-(1-propynyl)androsta-1,4,6-trione-3-one; Roussel-UCLAF, Romainville, France) to assess the responsiveness of the HPA axis to ether stress after chronic glucocorticoid suppression. Comparisons between the effects of DEX, which binds MR and GR (29–32), and RU 28362, which binds specifically to GR (33), could reveal the receptor type mediating these phenomena. OVX and E treatment were performed as described for Exp 1. Seventeen days after treatment, control and E-treated animals were each divided into three groups: animals received daily sc injections of DEX, RU 28362 (40 μg/100 g BW in oil; 0.2–0.3 ml), or oil for 4 days. Twenty-four hours after the last injection (day 21), rats were stressed for 1 min by exposure to ether vapor, as described in Exp 2. Blood samples (0.5 ml) were collected from the tail vein 0 min poststress, and trunk blood was collected after decapitation 20 min poststress. Plasma was removed and stored at −70 C until assayed for ACTH and CORT levels by RIA.

Exp 4: effect of E on MR and GR concentrations in the preoptic area (POA), medial basal hypothalamus (MBH), and HIPP. The studies described in this paper demonstrated that E elevates and prolongs the ACTH and CORT responses to stress in the female rat. This suggests an impairment of CORT negative feedback on the HPA axis, perhaps due to changes in specific populations of CORT receptors. Consequently, we measured MR and GR concentrations in various brain regions in control and E-treated rats. E treatment of adult female rats was performed as described in Exp 1. Twenty days after surgery, rats were ADX bilaterally. This surgical manipulation is necessary to deplete endogenous ligand (CORT) and allow occupied receptors to recycle to the unoccupied form, since occupied CORT receptors do not readily exchange their ligand with radiolabeled ligand. Eighteen hours after ADX, animals were killed, and the POA, MBH, and HIPP were dissected for measurement of MR and GR using an *in vitro* binding assay.

Exp 5: effect of E on the down-regulation of MR and GR. In this experiment we examined the ability of aldosterone (ALDO), RU 28362, and DEX to down-regulate MR and GR in the HIPP in the presence or absence of E. E treatment of adult female rats was performed as described in Exp 1. Twenty-one days after surgery, animals were bilaterally ADX. Control and E-replaced groups were subdivided into groups that received daily sc injections for 4 days of ALDO, RU 28362, or DEX (40 μg/100 g BW in oil; 0.2–0.3 ml) or oil. Animals were killed 24 h after the last injection. MR and GR were measured in the HIPP using an *in vitro* binding assay.

CORT receptor binding assays

MR and GR concentrations were determined using *in vitro* binding assays, as previously described (34). Briefly, tissue was homogenized in TEGMD buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 25 mM molybdate, and 1 mM dithiothreitol, pH 7.4), then centrifuged at 106,000 × *g* for 15 min at 40 C. One hundred-microliter aliquots of the supernatant cytosol were incubated with 5 nM [^{3}H]DEX or 5 nM [^{3}H]CORT in the presence of RU 28362 (to prevent binding to GR) for determinations of GR or MR, respectively. For GR measurements, 1 μM unlabeled RU 28362 (a GR specific agonist) was incubated in parallel tubes with [^{3}H]DEX to determine nonspecific binding. For MR measurements, 1 μM unlabeled DEX was incubated in parallel tubes with [^{3}H]CORT plus 1 μM unlabeled RU 28362 to assess nonspecific binding. DEX binds to both GR and MR, while RU 28362 binds only to GR. Therefore, the binding of [^{3}H]DEX in the presence of RU 28362 can be subtracted from the total [^{3}H]DEX binding to estimate binding to GR. DEX does not bind to CORT-binding globulin. This allows the concentration of MR to be determined by subtracting [^{3}H]CORT binding in the presence of DEX and RU 28362 from [^{3}H]CORT binding in the presence of RU 28362 alone. After incubations at 4 C for 16–20 h, samples were passed through Sephadex LH-20 columns to separate bound from free ligand. Six hundred microliters of eluate were collected that contained bound radioactivity. Three milliliters of UltimaGold (Packard, Downers Grove, IL) were added to the eluate, and radioactivity was counted in a Packard 1900 LA liquid scintillation counter (Downers Grove, IL) at 37% efficiency. All receptor data are expressed as femtomoles bound per mg protein. The method of Lowry *et al.* (35) was used to determine cytosolic protein concentrations.

Hormone assays

Blood samples were collected into tubes containing trasylol (1000 kallikrein inhibitor units) and EDTA. Plasma was removed and stored at −70 C until assayed for ACTH and CORT by RIA, as previously described (34, 36). ACTH plasma levels were determined using a double antibody RIA. The first antibody, directed against ACTH 7–18 (IgG, Inc., Nashville, TN), was used according to manufacturer's instructions. Standard curves were made using 0.5–50 pg/tube rat ACTH (Peninsula Laboratories, Belmont, CA). The intra- and interassay variabilities were 6% and 10%, respectively.

For CORT, binding proteins were heat denatured at 60 C for 1 h. Rabbit anti-CORT serum (Radioassay Systems Laboratories, Carson, CA) was used at a final dilution of 1:5600 according to manufacturer's protocols. Standard curves were constructed from dilutions of CORT (4-pregnen-11β,21-diol-3,20-dione; Steraloids, Wilton, NH). The intra- and interassay variabilities were 4.8% and 8.2%, respectively.

Plasma samples from DEX-, RU 28362-, and oil-injected rats used in Exp 5 were analyzed for residual hormone by a competitive *in vitro* binding assay. Briefly, plasma was incubated with [^{3}H]DEX and a supernatant cytosol from liver, as a source of MR and GR, prepared as described above. Standard curves were constructed using dilutions of DEX and RU 28362 combined with the liver cytosol and [^{3}H]DEX. Unbound radioactivity was removed using dextran-coated charcoal, and the bound radioactivity was counted as described above. The sensitivity of this assay was approximately 100 pg/ml.

Statistical analysis

Temporal differences in the ACTH and CORT responses to stress between control and E-treated rats were detected by two-way analysis of variance (ANOVA) with repeated measures across time, followed by the Student-Newman-Keuls multiple comparison test. Differences in percent suppression in Exp 3 were analyzed by the nonparametric Kruskal-Wallis *t* test. All other data were analyzed by a two- or three-way ANOVA, followed by the Student-Newman-Keuls multiple comparisons test (37).

Results

Exp 1a: effect of E on ACTH and CORT responses to footshock stress, a repeated sampling paradigm

Plasma samples obtained by repeated sampling from individual animals during their recovery from footshock stress showed significantly elevated CORT and ACTH levels in E-treated rats at 60 min ($P < 0.05$) *vs.* controls (Fig. 1). Two-way ANOVA with repeated measures across time revealed a significant time by treatment interaction ($P < 0.007$).

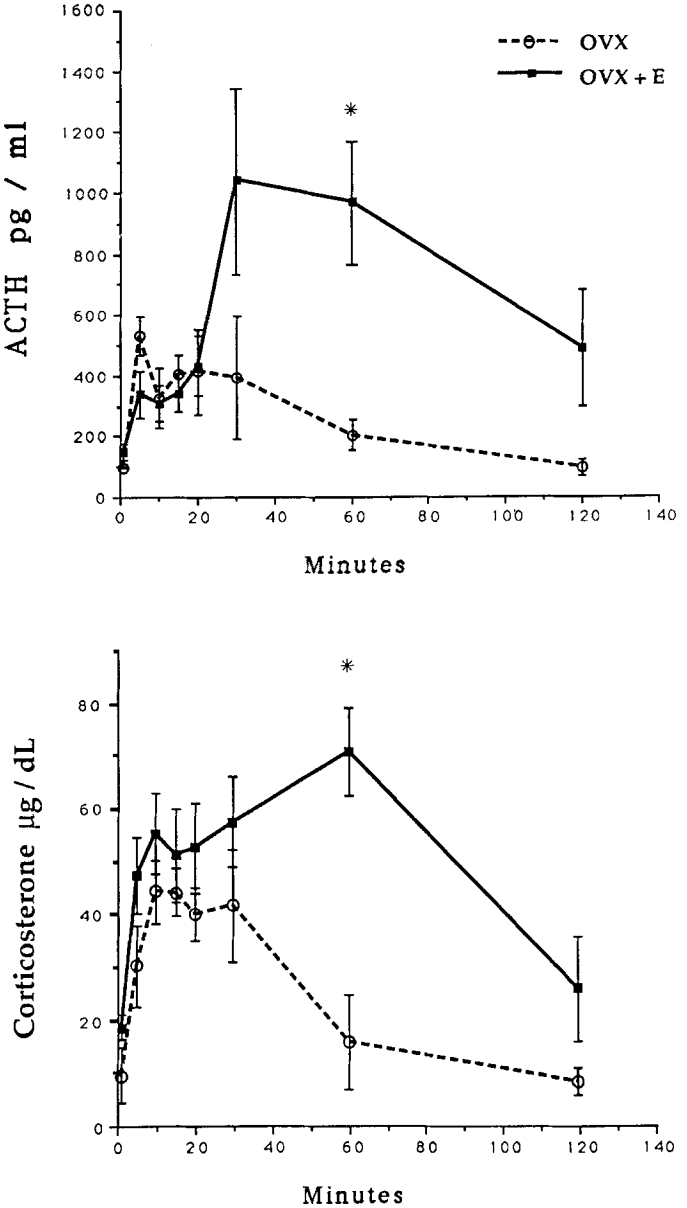

FIG. 1. Time course of plasma CORT and ACTH levels after footshock stress to female rats 21 days after OVX or OVX with E replacement (OVX + E). Serial blood samples were obtained by indwelling right atrial cannulae 0, 5, 10, 15, 20, 30, 60, and 120 min after 5 sec of footshock stress. Each *point* indicates the mean ± SEM of three to six determinations. Two-way ANOVA with one repeated measure across time indicated significant time and time by treatment differences ($P < 0.007$). *, Significant increase ($P < 0.03$) from OVX control value. The 5–30 min CORT values in the OVX group and the 5–60 min CORT values in the OVX + E group were significantly elevated ($P < 0.05$) over the 0 min control values. The 5, 15, 20, and 30 min ACTH values in the OVX group and the 30 and 60 min ACTH values in the OVX + E group were significantly elevated ($P < 0.05$) over the 0 min control values.

Exp 1b: effect of E on the CORT response to footshock stress, single point measurements

E treatment significantly elevated plasma CORT levels 0, 20, 45, and 70 min poststress ($P < 0.01$) compared to control values (Fig. 2). Two-way ANOVA revealed a significant time by treatment interaction ($P < 0.005$).

Exp 2: effect of E on the CORT response to ether stress

To further examine the effect of E on the HPA axis, a second stress paradigm using ether stress was performed. Twenty minutes after ether stress, CORT levels were significantly elevated ($P < 0.001$) in E-treated and control groups. However, E-treated animals had significantly greater plasma CORT levels 0 and 20 min poststress ($P < 0.05$) than the controls (Fig. 3).

Exp 3: effect of E on DEX- and RU 28362-mediated suppression of the neuroendocrine stress response

DEX and RU 28362 treatment of OVX control rats significantly depressed plasma ACTH and CORT levels ($P < 0.01$) when sampled 0 and 20 min after ether stress (Fig. 4). In the E-treated rats, DEX treatment significantly decreased the plasma ACTH and CORT response ($P < 0.01$); however, there was no significant effect of RU 28362 at 0 min. Ru 28362 was significantly less effective in suppressing the ACTH and CORT responses at 20 min in E-treated rats (69% and 78%, respectively) compared to those in OVX controls (39% and 23%, respectively; $P < 0.05$).

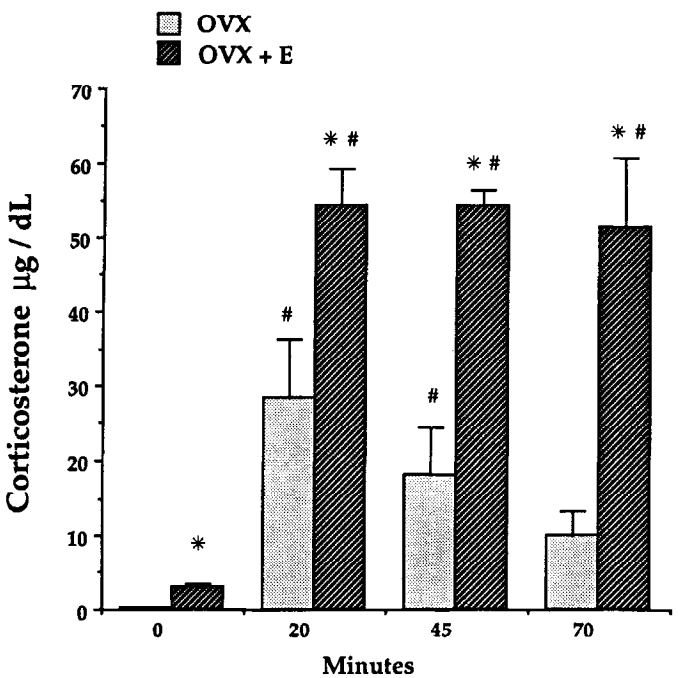

FIG. 2. Plasma CORT levels 21 days after OVX or OVX and continous treatment with E (OVX + E) are shown. Samples were obtained by decapitation 0, 20, 45, and 70 min after 5 sec of footshock stress. Each *bar* indicates the mean ± SEM of five or six determinations. Two-way ANOVA indicated significant time, treatment, and time by treatment differences ($P < 0.005$). *, Significant increase ($P < 0.03$) from OVX value. #, Significant elevation ($P < 0.05$) over 0 min control values.

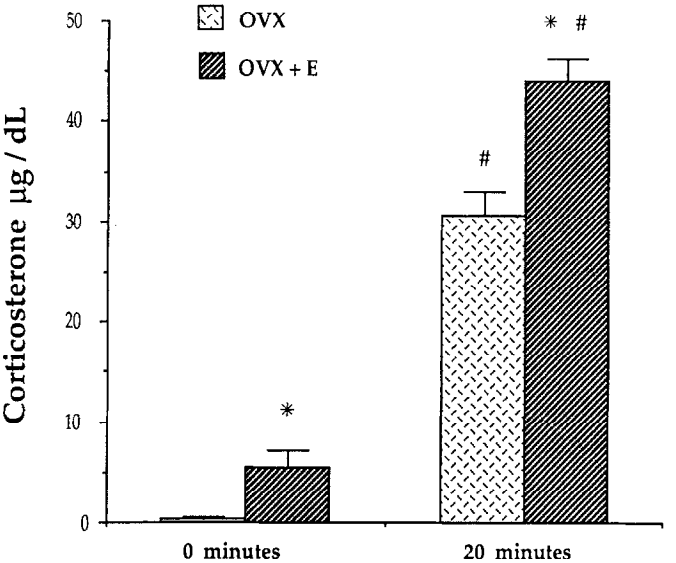

FIG. 3. Plasma levels of CORT in female rats 21 days after OVX or OVX with E capsule replacement (OVX + E). Samples were obtained at 0 and 20 min after 1-min exposure to ether vapors. Each *bar* indicates the mean ± SEM of five determinations. *, Significant increase ($P < 0.05$) from OVX value. #, Significant elevation ($P < 0.001$) over 0 min value.

Exp 4: effect of E on MR and GR concentrations in the POA, MBH, and HIPP

E treatment had no effect on the concentrations of MR or GR in any of the brain regions examined (Fig. 5). Scatchard analysis of hippocampal saturation binding data revealed apparent dissociation constants (K_d) of 0.5 and 0.6 nM for MR and 1.3 and 1.4 nM for GR in OVX and OVX + E groups, respectively.

Exp 5: effect of E on the down-regulation of MR and GR

To explore potential functional alterations in MR and GR, we examined the effect of E on the ability of ALDO, RU 28362, and DEX to down-regulate MR and GR in the HIPP.

Four days of either ALDO or DEX treatment significantly decreased the concentrations of MR and GR ($P < 0.05$) in the HIPP in E-treated and control groups (Fig. 6). Administration of the GR-specific agonist RU 28362 did not alter hippocampal MR levels in either group. RU 28362 treatment significantly decreased the concentration of hippocampal GR in the control group ($P < 0.05$), but not in the E-treated animals. Scatchard analysis of saturation binding data for the HIPP revealed no change in the K_d values (0.6 and 1.3 nM for MR and GR, respectively) in any of the treatment groups. Plasma samples obtained at the time of death from DEX- and RU 28362-injected animals were assayed and found to contain no residual glucocorticoid receptor-binding capacity.

Discussion

Previous studies have shown that female rats have higher basal plasma CORT levels as well as greater CORT responses

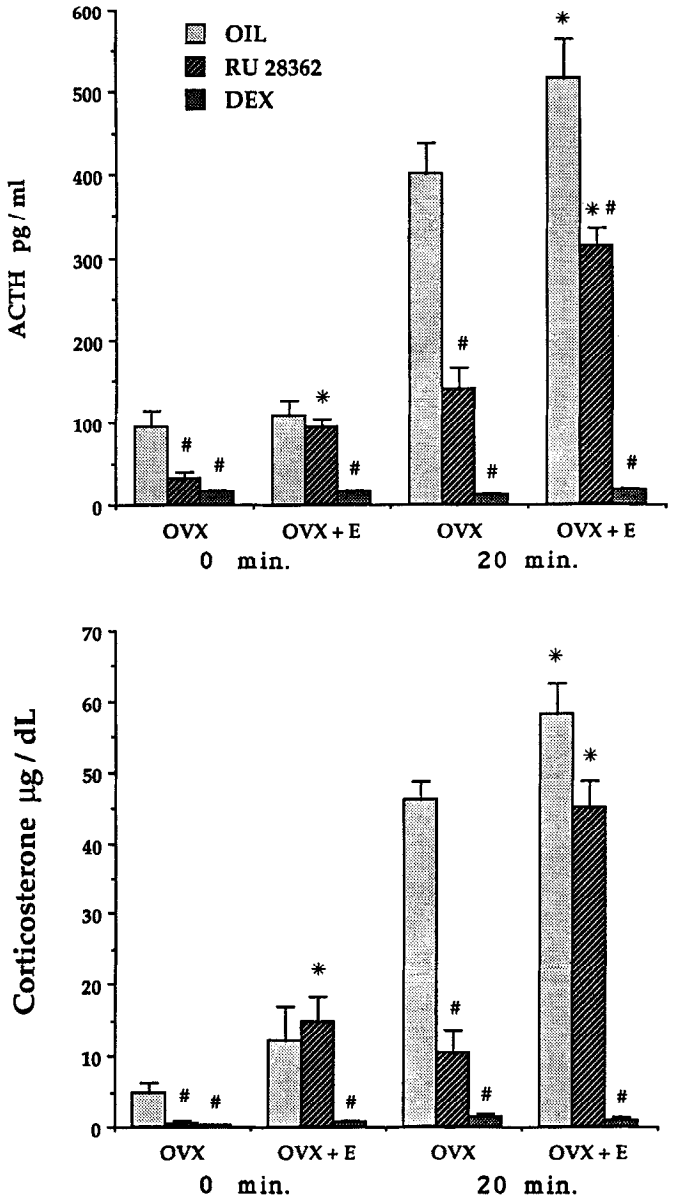

FIG. 4. Basal and poststress plasma levels of ACTH and CORT after RU 28362 or DEX treatment. Animals were OVX for 17 days or OVX with E replacement (OVX + E). Animals were subsequently sc injected daily for 4 days with either RU 28362 or DEX (40 µg/100 g BW in oil) or oil. Twenty-four hours after the last injection, plasma ACTH and CORT levels were measured 0 and 20 min after 1 min of ether stress. Each *bar* indicates the mean ± SEM of six determinations. Three-way ANOVA indicated significant treatment, injection, time, time by treatment, injection by treatment, and time by injection by treatment differences ($P < 0.03$). #, Significant decrease ($P < 0.01$) from oil control values. *, Significant difference ($P < 0.05$) from OVX control values.

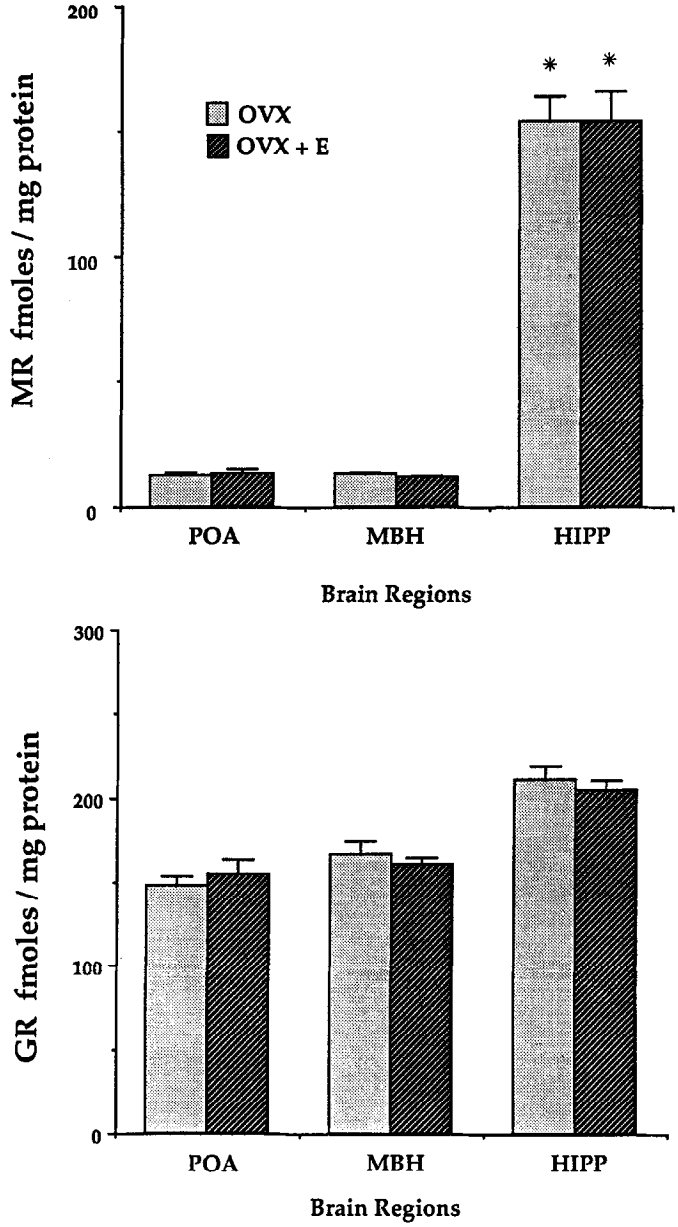

FIG. 5. Concentrations of MR and GR in the POA, MBH, and HIPP 21 days after OVX or OVX with E replacement (OVX + E). Each *bar* indicates the mean ± SEM of seven or eight determinations. *, Significant difference ($P < 0.001$) from other brain regions. No other significant differences in either receptor in any brain region examined were found.

to stress than males (1, 2). These sex differences appear to be estrogen mediated, since OVX of prepuberal female rats results in reduced basal plasma CORT levels, and E replacement reverses this change (3). In addition, OVX rats with E replacement have significantly higher poststress CORT levels than controls (5, 8). Our initial experiments were designed to test for this effect of E in our model system, using a chronic OVX female with or without E replacement. Phillips and Poolsanguan (5) demonstrated that chronic OVX itself does not alter either basal or stress-induced CORT levels. Our data show that basal and poststress CORT levels were higher in the presence of E, in agreement with these previous findings.

Since Kitay *et al.* showed that E can increase adrenal production of CORT directly (38), it is possible that the effects of E are mediated at the level of the adrenal gland. Therefore, we subsequently measured plasma ACTH as well as CORT during the recovery period after stress. The elevated and

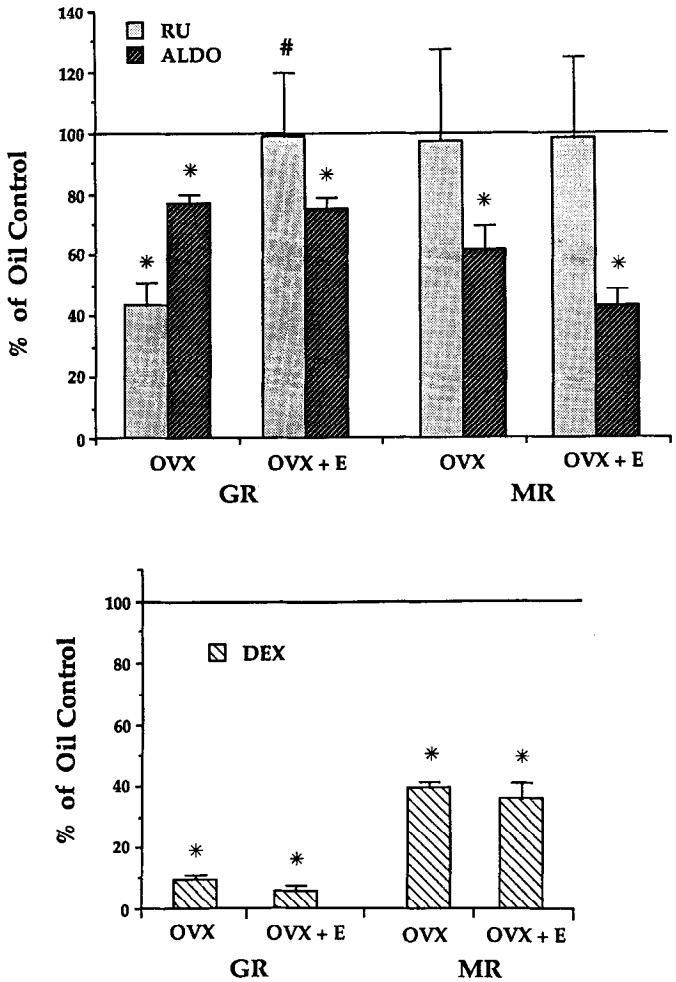

FIG. 6. Concentrations of GR and MR in the HIPP after ALDO or RU 28362 (A) or DEX (B) treatment. Animals were OVX for 17 days or OVX with E replacement (OVX + E). Animals were then ADX bilaterally and injected sc daily for 4 days with ALDO, RU 28362, or DEX (40 μg/100 g BW in oil), or oil. Each *bar* indicates the mean ± SEM of 6–10 determinations. *, Significant decrease ($P < 0.05$) from oil control. #, Significant difference ($P < 0.02$) from OVX value.

prolonged ACTH response to stress in the presence of E points to an effect at the level of the pituitary gland or higher. Examination of the time course of recovery provides additional information to help in elucidating the mechanism by which E produces its effects. These results clearly demonstrate an effect of E on ACTH secretion after two different types of physical stress. While an effect of E on the adrenal gland cannot be ruled out, our data demonstrate that there is a clear interaction of E at loci above the adrenal gland.

An impairment of CORT negative feedback could result in the pattern of hormonal secretion observed in the presence of E. Another possible explanation for the observed differences in ACTH and CORT secretion found in the presence of E is that E altered the clearance rate of the hormones. However, a recent study by Viau and Meaney (8), demonstrating elevated stress levels of ACTH and CORT in acutely E-treated animals compared to those in OVX controls, showed that the clearance of ACTH and CORT was comparable in both groups. In our hands, the basal levels of ACTH and CORT measured in cannulated animals were considerably higher than those in animals killed after removal from their home cages. This apparent discrepancy can be explained by the studies of Fagin *et al.* (39), who showed that even up to 1 week after cannulation, animals have elevated basal ACTH and CORT levels. However, our study demonstrating elevated poststress CORT levels in treated animals that were not cannulated is consistent with our observations of elevated CORT and ACTH levels in E-treated cannulated animals.

Importantly, not only the magnitude but also the duration of the CORT response to stress was increased in E-treated animals, resulting in an overall greater amount of circulating CORT during the 2 h after stress. Higher basal levels of CORT lead to greater occupancy of MR and GR, while higher stress levels lead to greater occupancy of GR (20, 26, 40). Correlations between CORT receptor occupancy and a variety of physiological end points, such as stress-induced CRF and vasopressin release (41), circadian peak ACTH secretion (42), and neuronal death (43), have been demonstrated. Thus, the overall amount of CORT "seen" by the CORT receptors appears to be a very important factor when considering the physiological consequences of CORT secretion, in contrast to examining the relative change from baseline.

The pattern of the ACTH response to stress we obtained is very similar to the pattern of the CORT response to stress obtained by Ratka *et al.* (26), who administered a GR antagonist before stress and subsequently examined CORT secretion. The antiglucocorticoid delayed the shut-off of the stress response, presumably by antagonizing CORT negative feedback. The similarity of the CORT secretory patterns in those studies and the present one strongly suggests that in the presence of E there is an impairment of the GR-mediated negative feedback.

We, therefore, assessed the responsiveness of the HPA axis after treatment with the synthetic glucocorticoids DEX and RU 28362. DEX and RU 28362 were administered to suppress the response of the HPA axis to stress. Since neither of these synthetic glucocorticoids is bound by CORT-binding globulins, and DEX binds to both MR and GR, while the specificity of RU 28362 is for GR, this study also provides an insight into the receptor specificity of E's actions. Our results demonstrate that E interferes with the ability of RU 28362, but not DEX, to suppress the ACTH and CORT responses to stress. This implies that E is interfering with GR-mediated slow negative feedback (18) and demonstrates that MR also plays a role in negative feedback. DEX binding to MR may directly effect slow negative feedback, or alternatively, DEX binding to MR may prevent E's effects from being manifested on GR. Support for a role for MR in mediating negative feedback has been provided by Ratka *et al.* (26), who showed that the pattern of the CORT response to stress was elevated and prolonged in the presence of a MR antagonist in a manner similar to that observed after the administration of a GR antagonist. In addition, Dallman *et al.* (25) showed that the IC_{50} values for inhibition of morning ACTH secretion by CORT and DEX are quite close to the reported K_d values for MR, implicating MR in the regulation

of basal ACTH levels. A more recent report by Bradbury *et al.* (42) using the MR antagonist spirolactone has implicated both MR and GR involvement in the inhibition of circadian peak ACTH secretion. These studies are consistent with our data showing an impaired delayed negative feedback action of glucocorticoids after E treatment, perhaps as a consequence of interference with GR-mediated signal transduction.

The differential effectiveness of RU 28362 and DEX in inhibiting CORT secretion could also be explained by a differential availability of RU 28362 and DEX to the regions involved in negative feedback. However, autoradiographic studies using [^{3}H]RU 28362 and [^{3}H]DEX have shown that both effectively label GR in the HIPP, POA, and other brain regions (33, 44, 45). Both ^{3}H-labeled ligands show uptake patterns similar to those obtained by immunohistochemistry using GR antibodies (46) or *in situ* hybridization using GR-specific probes (47). Consequently, we conclude that both ligands have equal access to the brain regions involved in mediating negative feedback. Whether DEX acts more effectively than RU 28362 at the level of the AP to inhibit stress-induced CORT secretion remains to be determined. The ability of DEX to bind to both MR and GR at saturating levels, while RU 28362 saturates only GR, strongly points to a selective effect of E on GR action.

The AP, hypothalamus, and HIPP have all been shown to be involved in CORT negative feedback of the HPA axis (18, 48, 49). These three regions also have been shown to contain high levels of GR and detectable to high levels of MR (22, 30, 50). The AP contains transcortin, an intracellular CORT-binding globulin that has a high affinity for CORT but a very low affinity for ALDO or DEX (19). Its presence in the AP complicates the distinction of MR from GR based on cytosolic binding assays. We have, therefore, limited our initial studies on the effects of E to brain regions that do not possess transcortin.

Negative feedback mechanisms involve CORT binding to MR and GR. Studies in aged male rats (15, 16) point to decreases in receptor number as the mechanism responsible for the decreased negative feedback and the increased hormonal response to stress. We, therefore, examined MR and GR to explore the possibility that E could be modulating CORT sensitivity by altering receptor concentrations.

Chronic E replacement (21 days) had no effect on MR or GR levels in any tissue examined, but this does not rule out any acute changes in receptor levels that may have normalized over time. These data are in agreement with a recent study that reported no change in hippocampal levels of MR or GR after 15 days of E treatment (51). In contrast, an earlier study had suggested that OVX resulted in an increase in hypothalamic CORT receptor levels, but receptor subtypes were not distinguished (52). Our findings suggest that the steady state levels of CORT receptor proteins are unaffected by chronic E treatment. Alternatively, E treatment may induce a posttranslational change in GR, such as a shorter half-life, as has been found after DEX treatment (53). This could mask changes in CORT receptor levels, since GR levels have been shown to rise 24 h after ADX (40). In either case, the functional efficacy of the CORT receptors is not addressed by binding studies. Rather, the *in vitro* binding assay employed to measure the levels of MR and GR relies only on the receptor's ability to bind ligand. While the levels of MR and GR appear unchanged, the capability of these receptors to regulate transcription remains undetermined.

Previous studies have shown that CORT receptors are autoregulated, with receptor stimulation decreasing receptor number, and ADX increasing it (30, 31, 40, 54). The down-regulation of these receptors was used in these studies to assess changes in CORT receptor function. Prior studies have found down-regulation of only GR by DEX in the rat (30, 40). In the mouse, however, down-regulation of both MR and GR by DEX treatment has been reported (31). It is of note, therefore, that we found DEX to down-regulate both GR and MR. Whether this is due to the use of female rats or differences in the binding assays remains to be determined.

Our findings, that E treatment interfered with the ability of RU 28362 to down-regulate GR while having no apparent effect on the ability of ALDO or DEX to down-regulate GR, have two distinct implications. First, it is evident that E affects predominately GR-mediated functions, such as the suppression of the CORT response to stress and down-regulation of GR. While RU 28362 accomplishes both of these functions in the OVX rat, it is significantly less effective in the presence of E. In addition, DEX is slightly more effective than RU 28362 in both functional assays. This may be due to DEX's binding to both MR and GR. The binding of DEX to MR could account for its increased efficacy compared to RU 28362, as both have nearly identical reported K_d values for GR (22). The lack of an effect of E on ALDO-induced GR down-regulation suggests that DEX-bound MR would also be unaffected by E.

Our results suggest that 1) the effects of DEX are being partially mediated through MR, and there may be interactions between DEX/MR- and DEX/GR-mediated signals; 2) the binding of DEX to MR may somehow prevent the action of E on GR; or 3) the effect of E on GR is limited to the functioning of RU 28362-bound GR, and DEX-bound GR can function unimpaired by E. Negative feedback and receptor down-regulation have both been thought to be GR mediated (22, 40). However, more recent studies suggest a role for MR (25, 26, 42) as well as the possibility of regulatory interactions between the receptor types (30, 55).

In summary, our findings have shown that E treatment elevates and prolongs activation of the HPA axis after two different physical stressors. Although there were no observable changes in MR or GR concentrations in E-treated rats, treatment with RU 28362, a GR-specific agonist, failed to suppress the CORT response to stress and autoregulate GR in the presence of E. E treatment interfered with two functions mediated by GR: receptor down-regulation and hormone suppression. These findings argue strongly for an effect of E on GR action and, perhaps, slow negative feedback as the mechanism by which E alters HPA function.

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