Age-Related Differences in Corticosterone Secretion in Female Rats

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The effects of age on steroidogenesis in rat zona fasciculata-reticularis (ZFR) cells were studied. Young, adult, and middle-aged rats were ovariectomized (Ovx) and received replacement therapy with oil or estradiol benzoate ([EB] 25 μg/mL/kg). Rat ZFR cells were incubated with corticotropin (ACTH), prolactin (PRL), or forskolin at 37°C for 1 hour. The effects of age on the activity of steroidogenic enzymes of ZFR cells were measured by the amount of intermediate steroidal products separated by thin-layer chromatography. Plasma levels were higher for PRL (54% to 254%) and corticosterone (179% to 257%) in middle-aged versus young rats. In oil-treated Ovx rats, basal and ACTH-stimulated corticosterone release by ZFR cells were also greater in middle-aged compared with young rats. Replacement with EB in Ovx rats increased the ACTH-stimulated release of corticosterone. Administration of ovine PRL in vitro resulted in a dose-dependent increase of corticosterone production. In oil-treated middle-aged rats, ovine PRL–stimulated corticosterone release was higher than in young rats. Forskolin-induced production of cyclic adenosine 3′,5′-monophosphate (cAMP) was greater in middle-aged versus young rats and correlated with the increase of corticosterone production. The activity of steroidogenic enzymes in rat ZFR cells was unchanged by age. These results suggest that the age-related increase of corticosterone production in female rats is associated with the stimulatory effect of PRL on ZFR cells and is due in part to an increase of cAMP generation.

VARIOUS REPORTS have shown that the basal level of plasma corticosterone is unchanged,[1,2] elevated,[3,4] or decreased[5] in aged animals. However, there is considerable disagreement as to the specific changes that occur, perhaps due to the use of different strains, animal models, sexes, or times or methods of sample collection. It has been shown that the steroidogenic capacity of the adrenals declines with age in rats, and this aging defect in corticosterone secretion lies distal to both corticotropin (ACTH) receptor binding and cyclic adenosine 3′,5′-monophosphate (cAMP) generation.[6] Popplewell and Azhar[7] have shown that the aging process increases the activity of mitochondrial side-chain cleavage in the adrenal.

In in vivo studies, ovariectomy decreases the synthesis and release of ACTH in the pituitary and the synthesis of corticosterone in the adrenal.[8,9] These effects are reversible by estradiol (E2) replacement and then increase both ACTH release and corticosterone secretion.[8] E2 receptors localized in brain regions may mediate hypothalamic-pituitary-adrenal (HPA) function,[10] and E2 is known to affect many elements of the HPA axis, including the neural input to corticotropin-releasing hormone (CRH) cells of the hypothalamic-hypophysial system, CRH synthesis and release,[11] corticotropic ACTH synthesis and secretion,[12] and glucocorticoid metabolism.[13]

It has been demonstrated that the activation of the HPA axis observed during the course of hyperprolactinemia may be explained by a direct stimulatory effect of prolactin (PRL) on both hypothalamic CRH and pituitary ACTH secretion.[14] The increased corticosterone secretion during hyperprolactinemia may be related to the increased secretion of ACTH, and a direct effect of PRL on adrenal steroidogenesis was reported.[15-17]

Numerous studies have examined the age-related changes of glucocorticoid secretion in males.[2,6,7] A few studies in females have shown patterns of age-related changes in glucocorticoid production similar to those in males.[18,19] However, the role of E2 in regulating the effects of age on adrenocortical function in female rats is not known. The involvement of cAMP production, a PRL effect, and changes in the activity of enzymes of steroidogenesis in the regulation of rat adrenocortical function during aging is also unclear. This study was undertaken to evaluate the role of E2 and PRL in regulating corticosterone secretion in ovariectomized (Ovx) rats during aging.

MATERIALS AND METHODS

Animals

Young (3 months), adult (6 months), and middle-aged (12 months) female Sprague-Dawley rats were bilaterally Ovx 2 weeks prior to experimentation. They were housed in a temperature-controlled room (22° ± 1°C) with 14 hours of artificial illumination daily (6 AM to 8 PM). Food and water were available ad libitum.

Effect of Age on Plasma PRL and Progesterone

Rat tail blood samples were collected between 9 and 10 AM under ether anesthesia. The plasma was separated and stored at −20°C. Plasma concentrations of PRL and progesterone were measured by radioimmunoassay (RIA).

Effects of Ovariectomy and E2 Replacement on Plasma PRL, Estradiol, and Corticosterone in Rats of Different Age Groups

Ovx rats were injected subcutaneously with sesame oil or estradiol benzoate ([EB] 25 μg/mL/kg body weight) once daily for 3 days before experimentation. The rats were then decapitated between 9 and 10 AM, the trunk blood was collected, and plasma samples were withdrawn, separated, and stored at −20°C. Plasma concentrations of PRL, estradiol, and corticosterone were measured by RIA.

Plasma (0.1 mL) was mixed with 1 mL diethyl ether (10× vol), shaken for 20 minutes, centrifuged at 1,000 × *g* for 5 minutes, and then quickly frozen in a mixture of acetone and dry ice. The organic phase was collected, dried, and reconstituted in a buffer solution (0.1% gelatin in phosphate-buffered saline, pH 7.5) before measurement of progesterone, estradiol, and corticosterone by RIA.

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Preparation of Zona Fasciculata-Reticularis Cells for Cell Culture and In Vitro Experiments

An adrenocortical preparation enriched with zona fasciculata-reticularis (ZFR) cells for culture was performed using a method previously described.[20] ZFR cells were incubated either with or without hormones dissolved in 1 mL per tube of Krebs-Ringer bicarbonate buffer (3.6 mmol K^+/L, 11.1 mmol glucose/L) with 0.2% bovine serum albumin (KRBGA medium) for 60 minutes at 37°C under 95% O_2 and 5% CO_2. To measure the effects of PRL and ACTH on corticosterone production in different age groups, ZFR cells were preincubated for 60 minutes with KRBGA medium and then incubated in tubes containing either 0.5 mL ovine PRL (1 to 100 nmol/L; Sigma, St Louis, MO) or ACTH (0.1 nmol/L; Sigma). To study the effects of age and EB replacement in vivo on the adenylyl cyclase and accumulation of cAMP in vitro, cells were again incubated for 60 minutes in a medium containing forskolin (1 μmol/L, Sigma). After priming with forskolin, the cells were incubated again for 60 minutes in tubes containing 0.5 mL forskolin. At the end of the incubation period, cells were homogenized in 500 μL 65% ice-cold ethanol by a polytron (PT-3000; Kinematica, Lucerne, Switzerland) and centrifuged at 200 × *g* for 10 minutes. The supernatants were lyophilized in a vacuum concentrator (SpeedVac; Savant Instruments, Holbrook, NY) and reconstituted with assay buffer (0.05 mol/L sodium acetate buffer with 0.01% azide, pH 6.2) before measuring the cAMP concentration by RIA.

RIA Procedures

PRL. The plasma PRL concentration was determined by RIA as described elsewhere.[21] Rat PRL-I-5 was used for iodination, and PRL-RP-3 was used as a standard preparation. The sensitivity of rat PRL RIA was 3 pg per assay tube. The intraassay and interassay coefficients of variation were 4.8% (n = 4) and 5.2% (n = 5), respectively.

Progesterone. The concentration of progesterone in plasma samples was determined by RIA as described elsewhere using antiprogesterone serum W-5.[22] The sensitivity of the progesterone RIA was 5 pg per assay tube. The intraassay and interassay coefficients of variation were 3.8% (n = 3) and 6.5% (n = 4), respectively.

Estradiol. The concentration of estradiol in plasma samples was determined by RIA as previously described using antiestradiol serum W-1.[23] The sensitivity of the estradiol RIA was 1 pg per assay tube. The intraassay and interassay coefficients of variation were 4.8% (n = 3) and 6.5% (n = 4), respectively.

Corticosterone. Plasma and media corticosterone concentrations were determined by RIA as described elsewhere[20] with anticorticosterone PSW#4-9. The sensitivity of the corticosterone RIA was 5 pg per assay tube. The intraassay and interassay coefficients of variation were 3.3% (n = 5) and 9.2% (n = 4), respectively.

cAMP. The concentration of adrenal cAMP was determined by RIA as described elsewhere.[20,22] With an anti-cAMP serum no. CV-27 pool, the sensitivity of the cAMP RIA was 2 fmol per assay tube. The intraassay and interassay coefficients of variation were 6.9% (n = 5) and 11.9% (n = 5), respectively.

Activity of 3β-Hydroxysteroid Dehydrogenase, 21β-Hydroxylase, and 11β-Hydroxylase

ZFR cells (50,000 cells per tube) were preincubated for 60 minutes at 37°C under 95% O_2 and 5% CO_2 in 1 mL KRBGA medium. After centrifugation at 200 × *g* for 10 minutes, the supernatant was discarded and the cells were incubated for 60 minutes in tubes with 0.2 mL KRBGA containing pregnenolone (10 nmol/L) and [^{3}H]pregnenolone (10,000 cpm, 5 pmol; NEN-DuPont, Boston, MA). After incubation, the media containing radioactive products were removed from the cultures by centrifugation at 200× *g* for 10 minutes. The media were extracted with diethyl ether (5× vol), shaken for 30 minutes, centrifuged at 200× *g* for 3 minutes, and then quickly frozen in a mixture of acetone and dry ice. The organic phase was collected, dried, and reconstituted in 100% ethanol. Aliquots of 50 μL of each sample and 5 μL unlabeled carrier steroids (1 mg/mL) were spotted onto silica gel G sheets containing fluorescent indicators (Macherey-Nagel, Düren, Germany) and chromatographed in a carbon tetrachloride:acetone (4:1 vol/vol) solution. The sheets were then dried, and steroid-containing spots were located under UV light. The migration rate (R_f) values were as follows: progesterone, 0.95; deoxycorticosterone (DOC), 0.7; and corticosterone, 0.3. The spots were then cut off and transferred to vials containing 1 mL liquid scintillation fluid (Ready Safe; Beckman Instruments, Fullerton, CA) before determination of the radioactivity in an automatic beta counter (Wallac 1409; Pharmacia, Turku, Finland).

In the experiment for incubation of ZFR cells with [^{3}H]pregnenolone, the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), 2β-hydroxylase, and 11β-hydroxylase was expressed as the radioactivity of [^{3}H]progesterone, [^{3}H]DOC, and [^{3}H]corticosterone, respectively.

Statistical Analysis

For the in vitro studies, the treatment mean values were tested for homogeneity using ANOVA, and the difference between specific means was tested for significance using Duncan's multiple-range test.[24] For analysis of EB effects, Student's *t* test was used. A difference between mean values was considered statistically significant at a P level less than .05.

RESULTS

Effects of Age on Plasma PRL and Progesterone in Intact Female Rats

The plasma level of PRL was higher ($P < .01$) in middle-aged rats (497 ± 90 ng/mL) versus young (144 ± 17 ng/mL) and adult (162 ± 12 ng/mL) rats. But the level of plasma progesterone was lower ($P < .01$) in middle-aged rats (39.4 ± 6.1 ng/mL) versus young rats (77.1 ± 8.6 ng/mL) (Fig 1).

Effects of Age and EB Replacement In Vivo

Plasma estradiol, PRL, and corticosterone in Ovx rats. Ovx rats were injected subcutaneously with EB (25 μg/mL/kg) once daily for 3 days. Plasma estradiol levels were 63.3 ± 11.1, 68.8 ± 15.1, and 60.9 ± 12.3 pg/mL in young, adult, and middle-aged EB-replaced Ovx rats (not shown). After ovariectomy, plasma PRL was higher in middle-aged and adult rats ($P < .05$ and $P < .01$, respectively) versus young rats. EB replacement resulted in an increase of plasma PRL in all age groups of Ovx rats. Plasma corticosterone was higher in both oil- and EB-replaced middle-aged Ovx rats versus young Ovx rats ($P < .01$). Plasma corticosterone in response to EB replacement was higher in all animals ($P < .05$ or $P < .01$) (Fig 2).

Spontaneous and ACTH-induced corticosterone release in vitro. Basal and ACTH (0.1 nmol/L)-stimulated release of corticosterone by ZFR cells was greater in oil-treated middle-aged Ovx rats (basal, 3.2 ± 0.7 ng/50,000 cells; ACTH, 54.5 ± 3.9 ng/50,000 cells) than in oil-treated young Ovx rats (basal, 0.9 ± 0.3 ng/50,000 cells; ACTH, 14.5 ± 2.5 ng/50,000 cells). After replacement of EB, the basal release of corticosterone in vitro in ZFR cells increased significantly in young ($P < .01$) and adult ($P < .05$) Ovx rats compared with the corresponding Ovx rats injected with oil (Fig 3). Replacement

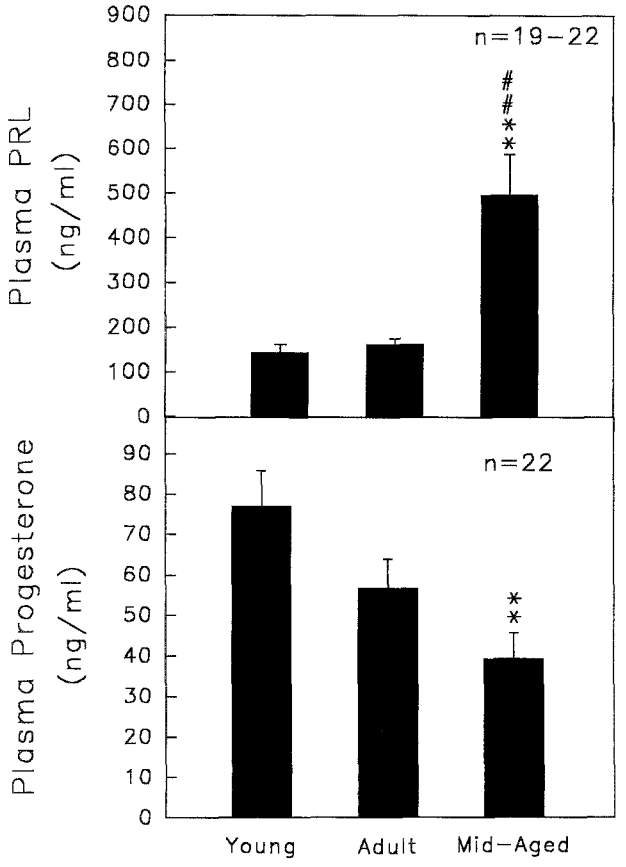

Fig 1. Effects of age on plasma PRL and progesterone concentrations in female rats. Tail blood samples were collected. **$P < .01$ *v* young rats. ##$P < .01$ *v* adult rats. Values are the mean ± SEM.

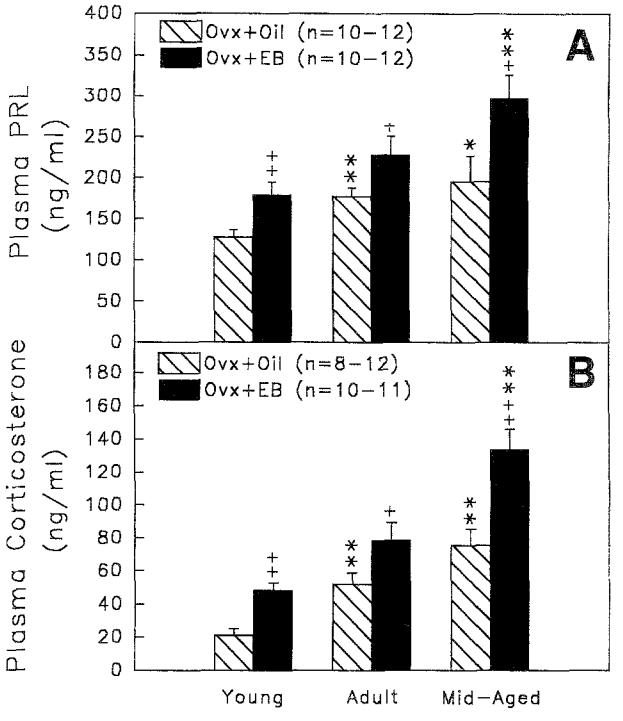

Fig 2. Effects of age and EB replacement on (A) plasma PRL and (B) corticosterone concentrations in Ovx rats. Female rats were Ovx 2 weeks before subcutaneous injection with either sesame oil, or EB (25 μg/mL/kg) once daily for 3 days. *$P < .05$, **$P < .01$ *v* young rats. †$P < .05$, ††$P < .01$ *v* Ovx + oil rats. Values are the mean ± SEM.

of EB in vivo resulted in an increase in ACTH-stimulated release of corticosterone in vitro in all groups. Corticosterone release in response to ACTH was higher in both EB- and oil-injected Ovx middle-aged rats compared with EB- and oil-injected Ovx young rats (Fig 3).

Corticosterone release and cAMP production in response to forskolin in vitro. Corticosterone release in Ovx oil- or EB-replaced rats from different age groups in response to forskolin (1 μmol/L) was tested for correlation. There was a positive correlation between corticosterone release and age ($P < .01$; Fig 4). cAMP production in ZFR cells in response to 1 μmol/L forskolin was higher (15.4 ± 3.4 fmol/50,000 cells, $P < .05$) in oil-treated middle-aged rats compared with oil-treated young rats (6.2 ± 1.1 fmol/50,000 cells). Forskolin-induced cAMP production in ZFR cells was not altered by EB replacement in Ovx rats. No aging effect on cAMP production was found in ZFR cells of Ovx rats following EB replacement (Fig 5).

Activity of 3β-HSD, 21β-hydroxylase, and 11β-hydroxylase. No age-related effects were found on the activity of 3β-HSD (conversion of [^{3}H]pregnenolone to [^{3}H]progesterone), 21β-hydroxylase (conversion of [^{3}H]pregnenolone to [^{3}H]progesterone and [^{3}H]DOC), and 11β-hydroxylase (conversion of [^{3}H]pregnenolone to [^{3}H]progesterone, [^{3}H]DOC, and [^{3}H]corticosterone) in oil- and EB-injected Ovx rats (Fig 6).

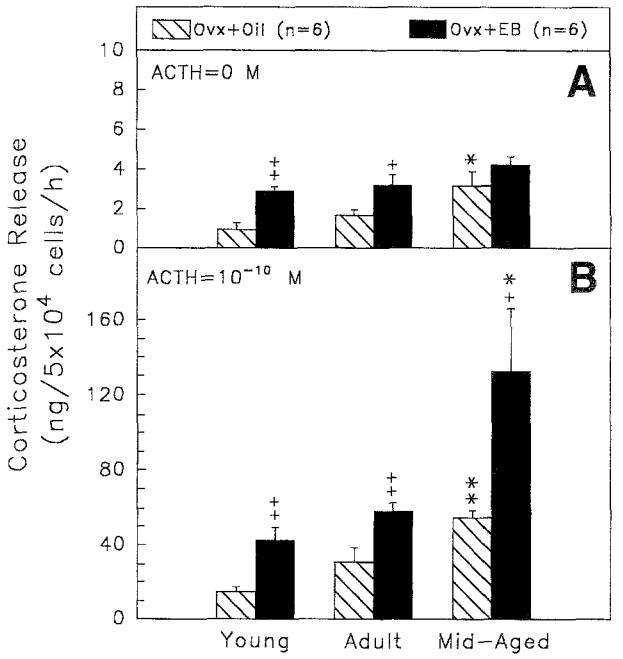

Fig 3. Effects of age and EB replacement on (A) basal and (B) ACTH (0.1 nmol/L)-stimulated release of corticosterone by ZFR cells of Ovx rats in vitro. See Fig 2 for details. *$P < .05$, **$P < .01$ *v* young rats. †$P < .05$, ††$P < .01$ *v* Ovx + oil rats. Values are the mean ± SEM.

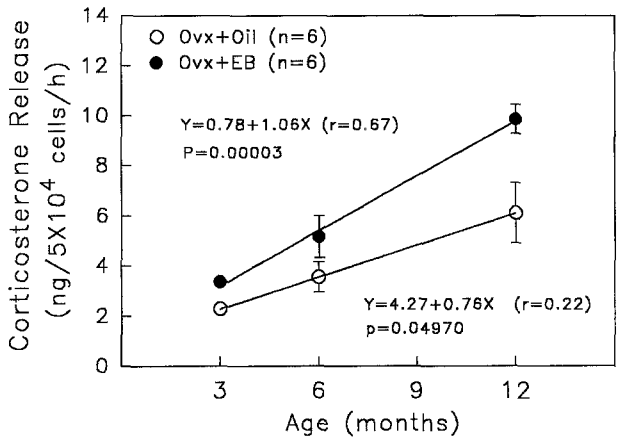

Fig 4. Regression analysis of the relationship between corticosterone release in response to forskolin and age in oil- or EB-replaced Ovx rats. Corticosterone release correlated with age in oil-replaced Ovx rats ($r = .22$, $P < .01$) and EB-replaced Ovx rats ($r = .67$, $P < .01$).

PRL-induced corticosterone release in vitro. Administration of ovine PRL (1 to 100 nmol/L) resulted in a dose-dependent increase in both oil- and EB-injected Ovx young, adult, and middle-aged rats. The release of corticosterone in response to 1 nmol/L PRL was higher ($P < .05$) in oil-treated middle-aged rats versus oil-treated young rats. No age effect on corticosterone release was observed in oil- or EB-injected Ovx rats after administration of a higher dose (ie, 10 or 100 nmol/L) of PRL. A stimulatory effect of EB on corticosterone release was found for PRL at a dose of 1 and 10 nmol/L, but not in ZFR cells treated with 100 nmol/L PRL (Fig 7).

DISCUSSION

The basal activity of the HPA axis tends to increase with age in rats, as evidenced by increased levels of plasma corticosterone.[18,19] These observations are in agreement with our in vivo and in vitro results, ie, corticosterone secretion increased with age. Several studies have reported that alterations in HPA axis function frequently accompany aging,[25] often resulting in an elevated secretion of glucocorticoids, which has been associated with cognitive impairment.[26] In older rats, the increased

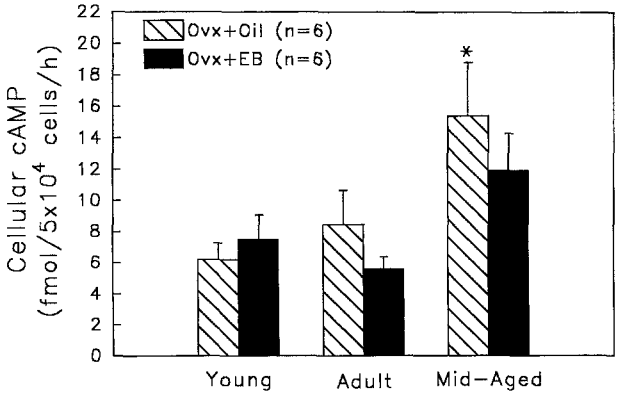

Fig 5. Effects of age and EB replacement in vivo on forskolin (1 μmol/L)-stimulated production of cAMP in ZFR cells of Ovx rats. *$P <$ .05 *v* young rats. Values are the mean ± SEM.

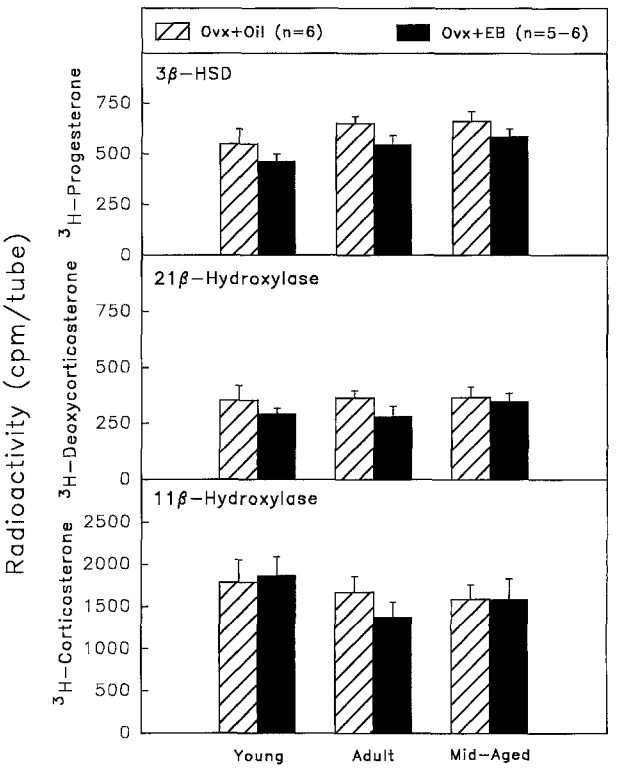

Fig 6. Effects of age and EB replacement in vivo on the activity of 3β-HSD, 21β-hydroxylase, and 11β-hydroxylase in ZFR cells of Ovx rats. Cells were incubated with 200 μL pregnenolone (10 nmol/L) and [³H]pregnenolone (5 pmol). Radioactive products in the medium were extracted with ether and then analyzed by thin-layer chromatography. Values are the mean ± SEM.

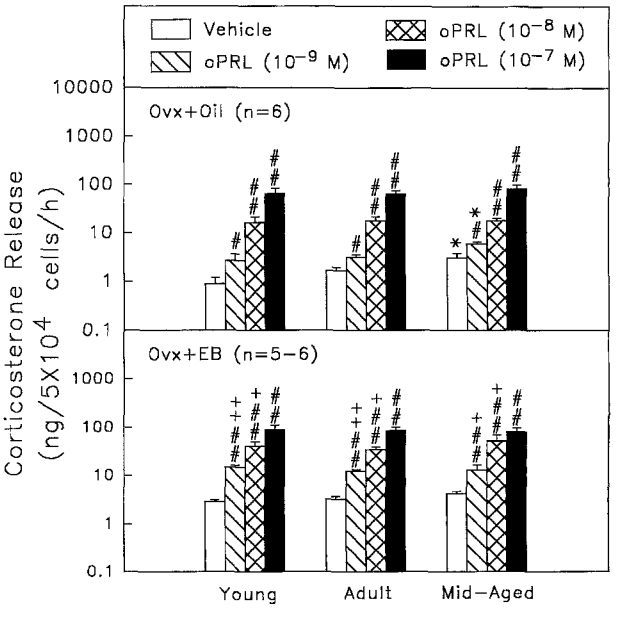

Fig 7. Effects of age and EB replacement in vivo on ovine PRL ([oPRL] 1-100 nmol/L)-stimulated release of corticosterone by ZFR cells of Ovx rats in vitro. *$P <$ .05 *v* young rats. †$P <$ .05, ††$P <$ .01 *v* Ovx + oil rats. #$P <$ .05, ##$P <$ .01 *v* oPRL 0 mol/L. Values are the mean ± SEM.

basal release of ACTH, presumably resulting from the hypothalamic CRH hypersecretion and down regulation of CRH receptors in the anterior pituitary gland,[27,28] is consistent with morphologic evidence of the hypertrophy of the ZFR area in the adrenal gland under chronic stimulation.[6,25,27,28]

In this study, we have explored the changes in plasma corticosterone concentrations and ZFR cell function in oil- and EB-treated Ovx rats of different ages. We demonstrated that plasma corticosterone increased age-dependently in both oil- and EB-treated Ovx rats. Similar to the observations for plasma corticosterone, both spontaneous and ACTH-stimulated corticosterone release by ZFR cells in oil-treated Ovx rats increased with age. Although EB replacement in vivo increased spontaneous and ACTH-stimulated corticosterone release in ZFR cells from both young and adult Ovx rats, administration of EB did not alter the spontaneous release of corticosterone by ZFR cells from middle-aged Ovx rats. These results imply that the stimulatory effect of estradiol on the spontaneous production of corticosterone by ZFR cells was prevented during aging.

It has been shown that E2 directly affects rat adrenocortical secretion, mainly by acting on the rate-limiting steps of steroidogenesis.[29] Our in vivo and in vitro results show that after replacement of EB for 3 days in Ovx rats, both the plasma corticosterone concentration and ACTH-stimulated corticosterone release from ZFR cells increased compared with oil treatment (Figs 2 and 3). These results are in agreement with previous reports in which E2 treatment increased the synthesis and release of ACTH and corticosterone.[12] E2 and progesterone regulate both stimulatory and inhibitory components of HPA activity.[30] Our data indicate that plasma progesterone was lower in middle-aged rats versus young rats. Furthermore, the inhibitory effects of progesterone on corticosterone secretion may be involved in the mechanism underlying the age-related alteration in corticosterone secretion.

A circadian rhythm for CRH secretion is thought to regulate the circadian pattern of ACTH and corticosterone secretion.[31] It has been shown that light-induced activation of suprachiasmatic nucleus neural activity is blunted in middle-aged rats.[32] Therefore, all rats in our experiment were killed at the same time, since we hoped to gain in vitro information on the intrinsic changes that may occur in these ZFR cells during aging. In the adrenal gland, initial events in ACTH action include hormone binding to the cell surface receptor followed by activation of the adenylyl cyclase–cAMP–protein kinase system.[33] Since corticosterone release and cAMP accumulation in response to forskolin were higher in ZFR cells from oil-treated middle-aged Ovx rats versus oil-treated young Ovx rats, the activity of adenylyl cyclase or phosphodiesterase might be increased or diminished by age (Fig 5). The prevention of an increased level of ZFR cAMP in middle-aged Ovx rats following E2 replacement may be due to an increase of phosphodiesterase activity caused by E2.

In the rat adrenal, corticosterone biosynthesis involves the active participation of cholesterol side-chain cleavage, 3β-HSD, 21β-hydroxylase, and 11β-hydroxylase activity.[34] Therefore, the age-related increase in corticosterone secretion was expected to have a profound effect on the activity of some or all steroidogenic enzymes. In the present study, we explored the possible relationship between steroidogenic enzyme activity and steroidogenesis in rat adrenal ZFR cells during aging. Our results indicate that steroidogenic enzyme activities were similar in all age groups.

It is well known that hyperprolactinemia occurs in older rats and activates the HPA axis.[35] Our data confirm that plasma PRL levels in middle-aged rats were higher than in young rats, either intact or Ovx. Administration of EB increased plasma PRL in rats of all ages. It has been shown that chronic hyperprolactinemia induced by tumor 7315b increases the hypothalamic content and the release of immunoreactive CRH.[36] This increased release of CRH in tumor-bearing rats is associated with an increase of plasma ACTH.[36] The present results indicate a positive correlation between the age-related increase of plasma PRL and plasma corticosterone.

Our in vitro study demonstrates that PRL 1 to 100 nmol/L stimulated the release of corticosterone by ZFR cells in all animals in a dose-dependent manner. Recently, the PRL receptor in rat and human adrenals has been described.[17,37] The direct effects of PRL on the release of corticosterone in ZFR cells are in agreement with previous observations.[16,17] Corticosterone release by ZFR cells in vitro in response to PRL (1 nmol/L) was higher in oil-treated middle-aged rats versus young rats. However, the E2- and age-dependent increases in corticosterone release were attenuated or abolished after incubation of ZFR cells with high doses of PRL (10 or 100 nmol/L). Inasmuch as the difference in corticosterone production between young and middle-aged rats can be prevented by a higher dose of PRL, the hypersecretion of corticosterone in female rats during aging is due in part to hyperprolactinemia. It has been shown that the Janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathway plays an important role in mediating the biological response to PRL in mammary epithelial cells.[38,39] Recently, we found that administration of PRL in vitro increased corticosterone release via an increase of cAMP production in ZFR cells (Chang LL, et al. unpublished data, October 1998). Although the mechanism by which PRL stimulates glucocorticoid production in ZFR cells has not been elucidated, the role of PRL in regulating the hypersecretion of glucocorticoids is interesting and worthy of exploration.

E2 activates rat PRL gene transcription involving the stabilization of a chromatin loop that facilitates protein-protein interactions between transcription factors.[40] It has been well established that either short-term (3 days) or long-term (30 days) administration of E2 in male rats significantly increases both plasma PRL and pituitary PRL.[41] The effects of estrogen on plasma and pituitary PRL could be secondary to the action of estrogen on hypothalamic PRL regulatory systems, including inhibition of dopamine release[42] and/or stimulation of serotonin,[43] or due to the direct effects of estrogen on pituitary lactotropes.[41]

In summary, we suggest that the hypersecretion of corticosterone in female rats during aging is due in part to (1) hyperprolactinemia and (2) increased accumulation of cAMP in response to decreased activity of phosphodiesterase in ZFR cells. However, the enzyme activities of steroidogenesis in ZFR cells are not altered by age.

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