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Effects of Estradiol on Aldosterone Secretion in Ovariectomized Rats

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Abstract The effects and action mechanisms of estradiol on aldosterone secretion in female rats were studied. Replacement of estradiol benzoate (EB) increased the levels of plasma estradiol and aldosterone in ovariectomized (Ovx) rats. The aldosterone release from zona glomerulosa (ZG) cells was higher in EB-treated rats than in oil-treated animals. EB treatment potentiated the responses of aldosterone release to adrenocorticotropic hormone (ACTH), forskolin (FSK), and 8-bromoadenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP). Administration of EB in vivo did not alter cAMP production in response to ACTH or FSK. Although angiotensin II (Ang II) increased aldosterone secretion by rat ZG cells, the stimulatory effect of Ang II on the release of aldosterone was not altered by EB treatment. The conversions of [^{3}H]-deoxycorticosterone to [^{3}H]-corticosterone and [^{3}H]-corticosterone to [^{3}H]-aldosterone in EB-treated groups were greater than those in the oil-treated group. These results suggest that estradiol increases aldosterone secretion in part through the mechanisms involving the activation of the post-cAMP pathway, 11β-hydroxylase and aldosterone synthase activity. J. Cell. Biochem. 73:137–144, 1999.

Key words: Ang II; ACTH; ZG cells; steroidogenesis; rat

Several investigators have reported that receiving oral contraceptives in women increases plasma renin activity (PRA) and plasma angiotensinogen concentration [Oelkers, 1996]. During pregnancy in humans [Weir et al., 1975] and in rats [Fowler et al., 1981], an increased PRA and plasma angiotensinogen concentration have also been reported. Sealey et al. [1994] have demonstrated that the increases in plasma renin and urinary aldosterone excretion are in coordination with increases in plasma estradiol and progesterone during ovarian stimulation in women.

Estrogen usage induced many physiological changes including alterations of vascular reactivity [Rosenfeld and Jackson, 1984; Naden and Rosenfeld, 1985; Vargas et al., 1995], sympathetic activity [Iversen, 1973] and the sodium balance [Johnson et al., 1972]. Specific binding sites of estrogen have been reported in aorta and cardiac ventricular tissues of both male and female rats [Stumpf et al., 1977; Lin and Shain, 1985]. Groh'e et al. [1996] have demonstrated that estrogen leads to both short- and long-term effects on the heart and may therefore account for gender difference in hypertensive heart disease. However, several controversial observations have been shown in the effects of estrogen on vascular reactivities. Administration of estradiol [Rosenfeld and Jackson, 1984; Naden and Rosenfeld, 1985] or progesterone [Hettiaratchi and Pickford, 1968; Nakamura et al., 1988] to various animal species produced attenuation of the vascular reactivity or pressor responsiveness to angiotensin II (Ang II). On the contrary, Vargas et al. [1995] demonstrated that estradiol potentiates the response to vasoconstrictor (norepinephrine) in rat isolated mesenteric preparation.

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Estrogen treatment has been shown to alter corticotropic releasing hormone (CRH) protein synthesis [Haas and George, 1989], adrenocorticotropic hormone (ACTH), and corticosterone release [Burgess and Handa, 1992] at multiple levels. Administration of 17β-estradiol in dispersed rat adrenocortical cells stimulated corticosterone secretion and steroidogenesis [Nowak et al., 1995]. It has been shown that estradiol increases adenosine 3′, 5′-cyclic monophosphate (cAMP) production in the pulmonary vascular smooth muscle cell [Farhat et al., 1996], immature rat uterine [Aronica et al., 1994], and human breast cancer cell [Aronica et al., 1994]. However, the effects of estradiol on the release of aldosterone and the action mechanisms in adrenal zona glomerulosa (ZG) cells are still uncertain.

Therefore, this study was designed to determine: 1) the effects of estradiol on plasma aldosterone; 2) the effects of estradiol on basal, Ang II-, and ACTH-stimulated release of aldosterone; 3) the mechanisms involved in the effects of estradiol on aldosterone release, including cAMP production and steroidogenesis of aldosterone in ZG cells of ovariectomized (Ovx) rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley female rats of 3 months old were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial illumination daily (0600–2000) and given food and water ad libitum.

Effects of Estradiol on Plasma Aldosterone in Ovariectomized (Ovx) Rats

Ovariectomy was performed under light ether anesthesia. Two weeks postovariectomy, female rats were subcutaneously injected with sesame oil (Sigma, St. Louis, MO) or estradiol benzoate (EB, 12.5, 25, or 50 μg/kg, Sigma) once daily for 3 days. After 3 days, female rats were decapitated in the morning, then the adrenal glands were rapidly removed and stored in a 0.9% (w/v) NaCl ice bath. To confirm the effects of ovariectomy and EB replacement, the uteri were weighed postdecapitation, then the concentration of plasma estradiol was measured by radioimmunoassay (RIA). The levels of plasma Na^+ and K^+ were determined by a flame photometer (EFOX 5053, Eppendorf, Hamburg, Germany).

The plasma was separated by centrifugation at 10,000 *g* for 1 min, then mixed with diethyl ether (10-fold the volume), shaken for 30 min, centrifuged at 1,000 *g* for 5 min, and quick-frozen in a mixture of acetone and dry ice. The organic phase was collected, dried, and reconstituted by a 1% bovine serum albumin (BSA) in borate buffer (pH 7.8) before measurement of the concentrations of aldosterone and estradiol by RIA.

Effects of Estradiol on the Basal, Ang II-, or ACTH-Stimulated Aldosterone Release in ZG Cells of Ovx Rats

The technique for the preparation of ZG cells was a modified method of Whitehouse and Abayasekara [1994]. Briefly, after removal of excess fat, the glands were separated into capsule (mainly ZG) and inner zone (mainly zona fasciculata/reticularis) fractions. The capsules from five to eight adrenal glands were assigned as one dispersion, then added to a polyethylene tube containing 1 ml Krebs-Ringer bicarbonate buffer with 3.6 mmol K^+/l, 11.1 mmol glucose/l and 0.2% BSA (KRBGA medium) and 2 mg collagenase (Sigma). The tube was aerated with 95% O_2 and 5% CO_2, then incubated for 1 h at 37°C in a shaker bath oscillating at 100 cycles per min. Generally, at least six dispersions (n = 6) of ZG cells were included in each group. At the end of incubation, the capsular tissues were mechanically dispersed into cells by repeated pipetting, then filtering through a nylon mesh. After centrifugation, cells were washed with deionized water for disrupting the red blood cells, then the osmolarity was immediately restored with 10-fold Hank's balanced sodium solution (HBSS). Finally, the cell pellets were resuspended in KRBGA medium. The cell number and cell viability (over 76%) were assessed by using a hemocytometer and the trypan blue exclusion method. The cells were preincubated with incubation medium for 1 h. The supernatant was decanted after centrifugation of the tubes at 200 *g* for 10 min. To determine the effects of estradiol replacement on the basal and the responses of aldosterone release to Ang II, ZG cells (5×10^4 cells) from oil- and EB (12.5, 25, or 50 μg/kg)-treated rats were incubated in polyethylene tubes for 1 h with 0.3 ml KRBGA medium or KRBGA containing Ang II (10^{-8} or 10^{-7} M, Sigma) at 37°C in a shaker bath (100 cycles per min) aerated with 95% O_2 and 5% CO_2. At the end of the incubation, 0.2 ml ice-cold KRBGA medium was added to stop the incubation. The medium was centrifuged at

200 *g* and stored at −20°C, until it was analyzed for aldosterone by RIA.

To assess the effects of estradiol on ACTH-stimulated aldosterone secretion and its involved mechanisms in aldosterone release from ZG cells, ZG cells were incubated with KRBGA, ACTH (2×10^{-9} M, Sigma), forskolin (5×10^{-6} M, an adenylyl cyclase activator, Sigma), or 8-Br-cAMP (10^{-4} M, a cAMP analog, Sigma) for 1 h in 0.3 ml of KRBGA medium. The medium was collected, then the cells from KRBGA, ACTH or forskolin groups were mixed with 0.5 ml of 65% ice-cold ethanol, next, homogenized by polytron (PT-3000, Kinematica Ag, Luzern, Switzerland), then centrifuged at 2,000 *g* for 15 min. The supernatants were lyophilized in a vacuum concentrator (Speed Vac, Savant, Holbrook, NY) and reconstituted with assay buffer (0.05 M acetate buffer with 0.01% sodium azide, pH 6.2) before measuring the concentration of cAMP by the RIA.

Effects of Estradiol Replacement on Postpregnenolone Steroidogenesis in ZG Cells of Ovx Rats

In order to study the effects of estradiol on the enzyme activity of postpregnenolone steroidogenesis in ZG cells, ZG cells were incubated with [^{3}H]-pregnenolone (10,000 cpm, 5 pmol, Amersham International plc, Buckinghamshire, UK) for 1 h. The medium was collected, extracted by diethyl ether, then quick frozen in a mixture of acetone and dry ice. The diethyl ether layer was collected, dried, and reconstituted in 100 µl 100% ethanol containing 5 µg of each of the carriers, including progesterone (P_4), deoxycorticosterone (DOC), corticosterone, and aldosterone. Aliquots (50 µl) of samples were applied to a thin layer chromatography plate (0.25 µg thick silica gel G sheets precoated with fluorescent indicator, Macherey-Nagel, Düren, Germany) then developed in a mixture of carbon tetrachloride and acetone (4:1; vol/vol). The sheets were dried and the location of steroid-containing spots were indicated under u.v. light. The R_f values were 0.97 for P_4, 0.68 for DOC, 0.24 for corticosterone and 0.11 for aldosterone. The spots were cut off and transferred into vials containing 1 ml of liquid scintillation fluid (Beckman, Fullerton, CA) before the radioactivity was counted in an automatic beta counter (Wallac 1409, Pharmacia, Turku, Finland).

RIA of Aldosterone

The anti-aldosterone antiserum No. 088, provided by the National Institute of Health (NIH, Bethesda, MD) was diluted with 1% BSA-borate buffer. The cross-reactivities of anti-aldosterone were less than 1% with cortisol, corticosterone, cortisone, testosterone, dehydroepiandrosterone, P_4, estradiol, 18-hydroxycorticosterone, and DOC. In this RIA system, a known amount of unlabeled aldosterone, an aliquot of plasma extract or medium samples, adjusted to a total volume of 0.3 ml by a buffer solution (1% BSA-borate buffer, pH 7.8), was incubated with 0.1 ml aldosterone antiserum (1:120,000 dilutions) diluted with 1% BSA-borate buffer and 0.1 ml [^{3}H]-aldosterone (approximately 8,000 cpm, Amersham) at 4°C for 24 h. Duplicate standard curves with five points ranging from 3 to 800 pg of aldosterone were included in each assay. An adequate amount (0.2 ml) of 0.5% dextran-coated charcoal (Sigma) was added and further incubated in an ice bath for 15 min. After incubation, the assay tubes were centrifuged at 1,000 *g* for 15 min. The supernatant was mixed with 3 ml liquid scintillation fluid (Beckman, Fullerton, CA) before counting the radioactivity in an automatic beta counter (Wallac 1409, Pharmacia, Turku, Finland). The sensitivity of aldosterone RIA was 4 pg per assay tube. The intra- and interassay coefficients of variation were 7.5% (n = 5) and 8.1% (n = 5). The inhibition curves produced by ether-extracted rat plasma and the incubation medium of rat ZG cells were parallel to those given by unlabelled aldosterone.

RIA of Estradiol

The concentration of plasma estradiol was determined by RIA as described elsewhere with anti-estradiol serum no. W1 [Lu et al., 1998]. The sensitivity of estradiol RIA was 1 pg per assay tube. The intra- and interassay coefficients of variation were 6.0% (n = 5) and 5.9% (n = 5), respectively.

RIA of cAMP

The concentration of cAMP in ZG cells extracted by ethanol was measured by an RIA developed in our laboratory as described elsewhere [Chen et al., 1997; Lo et al., 1998]. The sensitivity of cAMP RIA was 2 fmol/assay tube. The anti-cAMP CV-27 pool was provided by the National Institute of Diabetes and Digestive

and Kidney Diseases (NIDDKD). The synthetic tyr-cAMP (Sigma, St. Louis, MO) used for iodination and the unlabelled cAMP (Sigma) which served as a standard preparation. The sensitivity of cAMP RIA was 2 fmol per assay tube. The intra- and interassay coefficients of variability were 3.8% (n = 4), and 6.6% (n = 5), respectively.

Statistical Analysis

All the data were expressed as mean ± SEM. The treatment means were tested for homogeneity using an analysis of variance, and the difference between specific means was tested for significance using Duncan's multiple-range test [Steel and Torrie, 1960]. A difference between two means was considered to be statistically significant when P was less than 0.05.

RESULTS

Effects of Estradiol on Plasma Aldosterone in Ovx Rats

Ovx rats that were replaced with different doses of EB (12.5, 25, and 50 μg/kg), gradually increased their uterine weights (338 ± 10, 342 ± 25, 350 ± 19 mg), as compared with oil-treated (176 ± 11 mg) Ovx rats. Replacement with different doses of EB induced an increase in the levels of plasma estradiol in a dose-dependent manner ($P < 0.05$ or $P < 0.01$, Fig. 1) in Ovx rats. EB at 25 and 50 μg/kg produced a marked increase ($P < 0.05$ and $P < 0.01$) in plasma aldosterone in Ovx rats (Fig. 1). Plasma concentrations of Na^+ (from 139 ± 3 to 142 ± 2 mEq/l) and K^+ (from 3.9 ± 0.2 to 4.0 ± 0.1 mEq/l) had no significant differences among oil- and EB-treated rats.

Effects of Estradiol on the Basal, Ang II- or ACTH-Stimulated Aldosterone Release in ZG Cells from Ovx Rats

The basal (unstimulated) release of aldosterone by ZG cells in EB (25, 50 μg/kg)-treated rats was higher ($P < 0.01$) than in oil-treated Ovx rats (Fig. 2). Ang II (10^{-8} M or 10^{-7} M) markedly ($P < 0.01$, Fig. 2) increased aldosterone release by ZG cells as compared with the level of the vehicle group (basal release) in oil-treated rats. In EB-treated groups, Ang II (10^{-8} M) failed to produce significant increase in aldosterone release until the administration of Ang II (10^{-7} M, $P < 0.05$, Fig. 2).

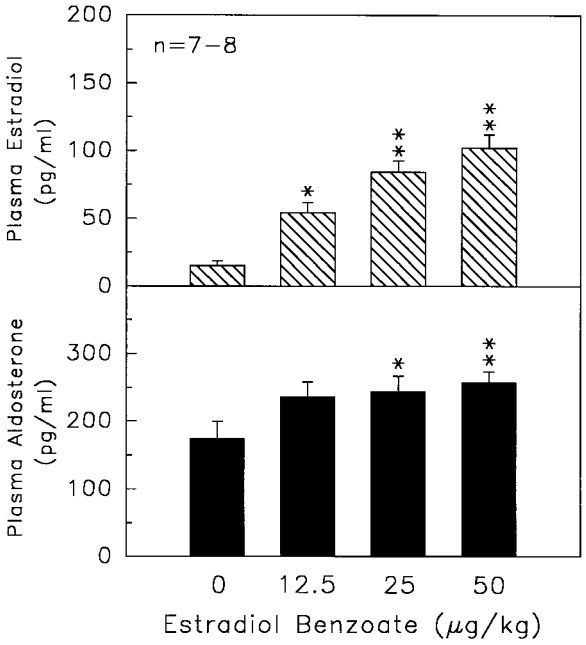

Fig. 1. Effects of estradiol benzoate replacement on the concentrations of plasma estradiol and aldosterone in ovariectomized (Ovx) rats. *, **, $P < 0.05$, $P < 0.01$ as compared with oil (EB = 0 μg/kg)-treated rats, respectively. Each value represents mean ± SEM.

Administration of ACTH (2×10^{-9} M), forskolin (5×10^{-6} M) or 8-Br-cAMP (10^{-4} M) produced a significant ($P < 0.05$ or $P < 0.01$) increase in aldosterone release from ZG cells (Fig. 3) in both oil- and EB-treated rats. ACTH increased aldosterone release by 1.4 ± 0.1, 1.8 ± 0.4, 1.7 ± 0.2 and 2.4 ± 0.4 ng/5 × 10^4 cells/h in ZG cells from the oil- and EB-treated rats, respectively. Forskolin increased aldosterone release by 0.6 ± 0.1, 1.2 ± 0.3, 1.1 ± 0.2, and 1.8 ± 0.2 ng/5 × 10^4 cells/h from the oil- and EB-treated rats, respectively. By comparison, these net increments of aldosterone production demonstrated that the EB (50 μg/kg)-treated group had a greater response to ACTH or forskolin than those in the oil-treated group. As compared with the oil-treated group, 8-Br-cAMP produced a higher aldosterone secretion in the EB (50 μg/kg)-treated group (Fig. 3, bottom). Administration of ACTH or forskolin significantly increased the accumulation of cAMP in ZG cells of both the oil- and the EB-treated rats (Fig. 4). However, no significant differences in the basal, ACTH- or forskolin-stimulated cAMP accumulation among oil- and EB-treated rats were observed.

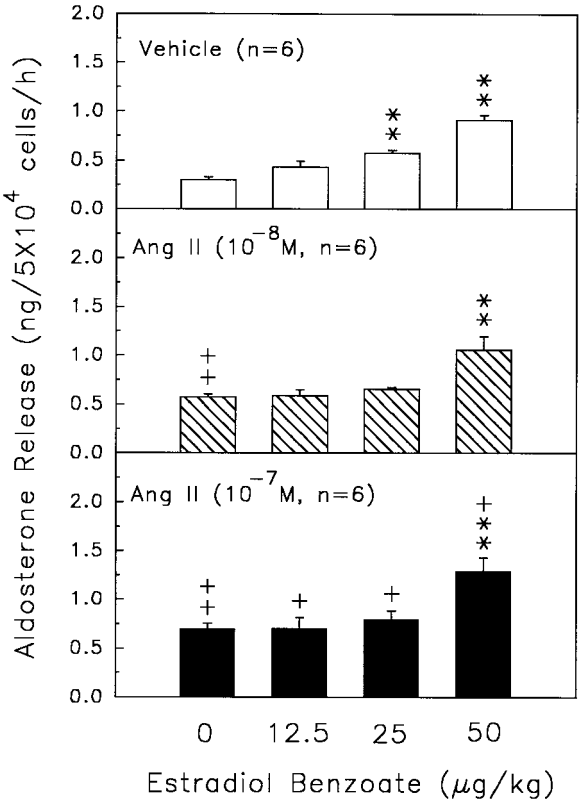

Fig. 2. Effects of estradiol benzoate (EB, 12.5, 25, or 50 μg/kg) replacement on the basal (vehicle), and Ang II (10^{-8}, 10^{-7} M)-stimulated aldosterone release by ZG cells in Ovx rats. **, $P < 0.01$ as compared with oil-treated rats. +, ++, $P < 0.05$, $P < 0.01$ as compared with the vehicle group, respectively. Each value represents mean ± SEM.

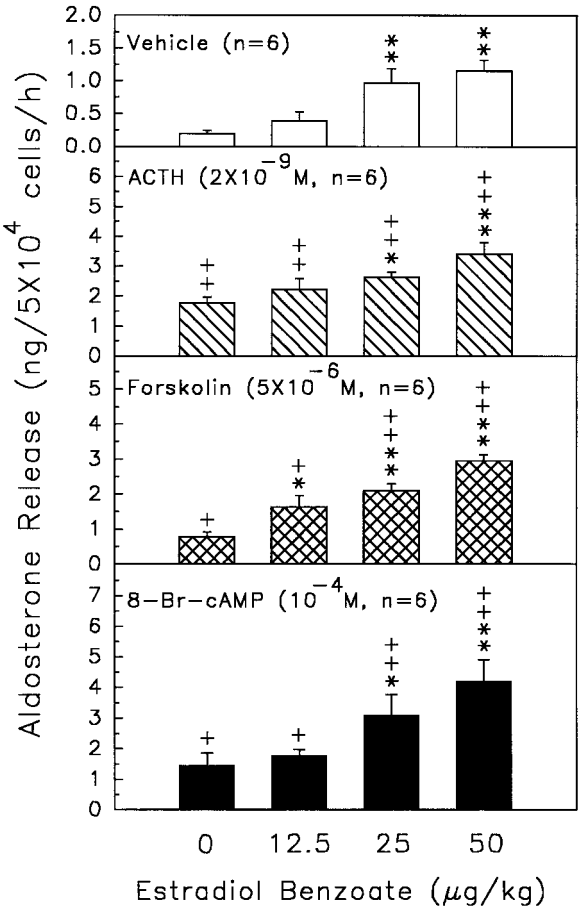

Fig. 3. Effects of estradiol benzoate (EB, 12.5, 25, or 50 μg/kg) replacement on the basal, ACTH (2×10^{-9} M)-, forskolin (5×10^{-6} M)-, and 8-Br-cAMP (10^{-4} M)-stimulated aldosterone release by ZG cells in Ovx rats. *, **, $P < 0.05$, $P < 0.01$ as compared with oil-treated rats, respectively. +, ++, $P < 0.05$, $P < 0.01$ as compared with the vehicle group, respectively. Each value represents mean ± SEM.

Effects of Estradiol on the Enzyme Activity of Postpregnenolone Steroidogenesis in ZG Cells from Ovx Rats

Incubation of ZG cells with [^{3}H]-pregnenolone for 1 h resulted in the formation of [^{3}H]-P_4, [^{3}H]-DOC, [^{3}H]-corticosterone, and [^{3}H]-aldosterone (Fig. 5). EB treatment produced a decreased [^{3}H]-DOC accumulation and an increased [^{3}H]-aldosterone production. There were no significant differences in [^{3}H]-P_4 and [^{3}H]-corticosterone accumulation among those four groups. It has been well known that aldosterone synthase is responsible for the conversion of corticosterone to aldosterone. The 11β-hydroxylase is responsible for the conversion of DOC to corticosterone. Therefore, we suggested that the activities of aldosterone synthase and 11β-hydroxylase were increased by EB treatment.

DISCUSSION

The present study demonstrated that estradiol replacement increases the level of plasma aldosterone and basal release of aldosterone by ZG cells in Ovx rats. The effects of estradiol on steroidogenesis in the adrenal cortex have been shown. Nowak et al. [1995] have suggested that estradiol increased corticosterone secretion is due to the increased conversion of cholesterol to pregnenolone in rat adrenocortical cells. In our study, the conversions of the [^{3}H]-corticosterone to [^{3}H]-aldosterone and the [^{3}H]-DOC to [^{3}H]-corticosterone in the EB-treated groups were higher than in the oil-treated group, which suggests that the activities of aldosterone synthase and 11β-hydroxylase were increased by the administration of estradiol.

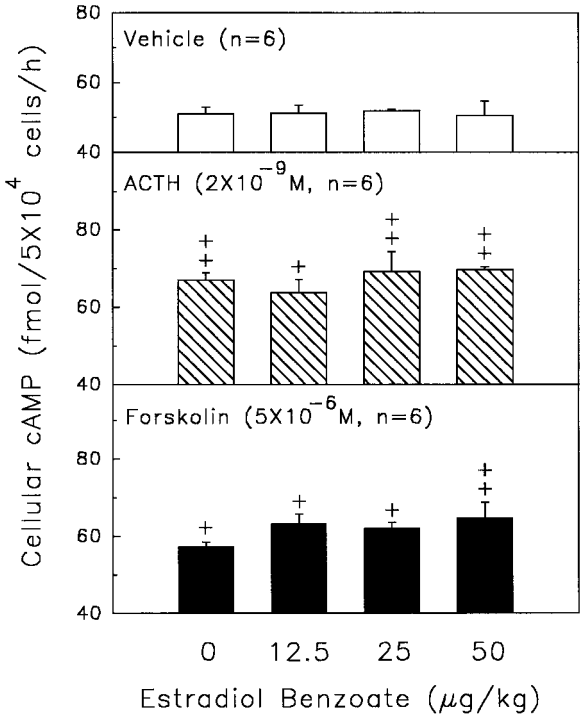

Fig. 4. Effects of estradiol benzoate (EB, 12.5, 25, or 50 μg/kg) replacement on the basal, ACTH (2×10^{-9} M)-, and forskolin (5×10^{-6} M)-stimulated cAMP production in ZG cells from Ovx rats. +, ++, $P < 0.05$, $P < 0.01$ as compared with the vehicle group, respectively. Each value represents mean ± SEM.

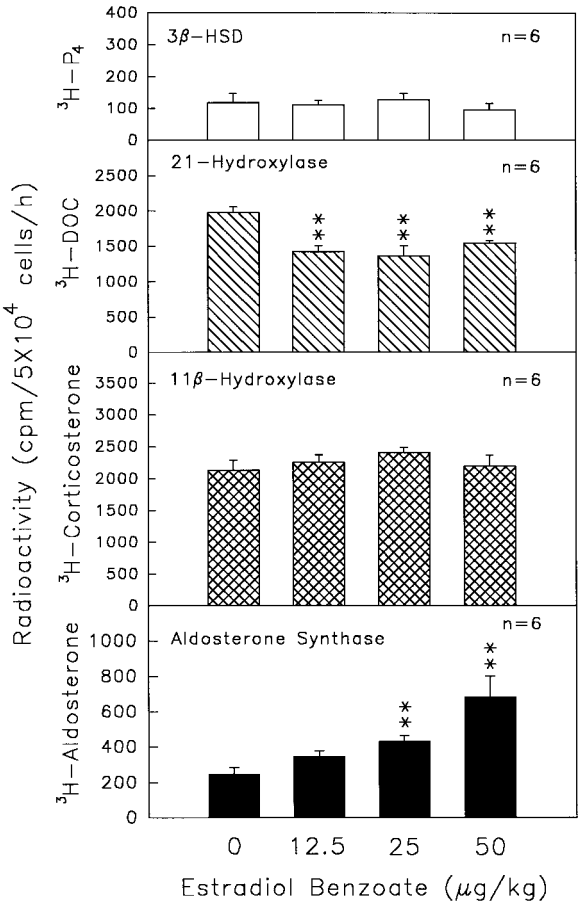

Fig. 5. Effects of estradiol benzoate (EB, 12.5, 25, or 50 μg/kg) replacement on the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD), 21-hydroxylase, 11β-hydroxylase, and aldosterone synthase in ZG cells from Ovx rats. Rat ZG cells were incubated with ^{3}H-pregnenolone (10,000 cpm) at 37°C for 1 h. Radioactive products in the medium were extracted by ether and then analyzed by thin layer chromatography. **, $P < 0.01$ as compared with oil-treated rats. Each value represents mean ± SEM.

Several studies have indicated that estradiol may directly affect the intracellular cAMP through activation of the adenylyl cyclase [Aronica et al., 1994; Farhat et al., 1996]. For example, short-term exposure to estradiol increased both basal and forskolin-stimulated cAMP levels in pulmonary vascular smooth muscle cells [Farhat et al., 1996]. However, in our study, estradiol treatment did not produce changes in basal, ACTH- or forskolin-stimulated cAMP production. Therefore, these results strongly suggest that cAMP production is not involved in the estradiol-enhanced secretion of aldosterone in ZG cells. However, after administration of ACTH, forskolin, or 8-Br-cAMP, a greater increase in aldosterone release was shown in the EB (50 μg/kg)-treated group. Therefore, we suggest that the activation of the post-cAMP pathway definately plays an important role in the increase of aldosterone release in estradiol-treated rats.

Hypertension occuring in women taking oral contraceptives or estrogens has been ascribed to increase in plasma angiotensinogen concentration [Oelkers, 1996]. It has been shown that estrogens directly increase the synthesis and secretion of angiotensinogen in hepatocytes [Klett et al., 1992] and angiotensinogen mRNA in rat liver [Klett et al., 1993]. In this study, we demonstrated that estradiol treatment in vivo increases aldosterone production at the level of adrenal ZG cells. In addition, we found that estradiol treatment did not alter the responses of aldosterone release to Ang II in ZG cells. The attenuation of responsiveness to Ang II has been demonstrated in other sites. In vascular system, treatment with estrogen reduced the vascular reactivity or pressor response to Ang II [Rosenfeld and Jackson, 1984; Naden and Rosenfeld, 1985]. The increased prevalence of hypertension and cardiovascular morbidity af-

ter menopause suggests that the estrogen might exert a protective effect on the cardiovascular system [Weiss, 1972]. Conversely, oral contraceptives can induce some metabolic changes similar to those associated with an increased risk of coronary heart disease, including raised serum triglyceride, low-density lipoprotein (LDL) cholesterol, and decreased high-density lipoprotein (HDL) cholesterol levels [Wynn et al., 1966, 1979]. Taken together, these results suggest that the effects of estrogen on the renin-angiotensin-aldosterone system is involved in the maintenance of body fluid balance, and thereby permits the physiologically required fluid or electrolyte expansion during pregnancy. Although the effects of estrogen on cardiovascular responsiveness are still controversial, these findings provide the important consideration in postmenopausal estrogen therapy and the etiology of blood pressure elevation with oral contraceptives.

The level of plasma prolactin (PRL) significantly increased after chronic treatment with estradiol (data not shown). This result is in agreement with previous studies that estrogen stimulates PRL release, both in vivo [Terry et al., 1985] and in vitro [Ben-David et al., 1964; Nicoll and Meites, 1964]. The role of PRL in adrenal function and steroid hormone secretion has been studied. Mazzocchi et al. [1986] found that chronic PRL treatment induced hypertrophy of ZG cells and an increase in the blood level of aldosterone in rats. Recently, we have reported that administration of PRL in vitro stimulated aldosterone release by rat ZG cells [Kau et al., 1999]. The physiological significance of PRL involved in the regulation of aldosterone secretion probably is attributed to the increase of blood volume and body fluid turnover during pregnancy. Base on these observations, we suggest that PRL may play a role in the effect of chronic treatment with estradiol on the secretion of aldosterone. However, additional studies are needed to support this hypothesis that PRL mediates the stimulatory effects of estradiol on aldosterone secretion.

In summary, estradiol treatment in vivo increased aldosterone secretion. This estradiol-induced aldosterone secretion was independent of cAMP production. The activation of the post-cAMP pathway, 11β-hydroxylase, and aldosterone synthase activity were involved in the increase of estradiol on aldosterone secretion.

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