Estrogen Regulates Adrenal Angiotensin Type 1 Receptors by Modulating Adrenal Angiotensin Levels

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Estrogen inhibits adrenal angiotensin type 1 receptor (AT1R) binding sites and attenuates the adrenal responsiveness to angiotensin II (Ang II). Ang II modulates AT1R expression. Here, we determined if estrogen-induced down-regulation of adrenal AT1Rs involves modulation of adrenal Ang II. Female rats were ovariectomized (OVX) and injected with 17β-estradiol (E2; 40 μg/kg) or vehicle for 7 d. Adrenal Ang II was separated from other angiotensin peptides by HPLC and measured by RIA. Scatchard analysis of radioligand binding curves showed that E2 or captopril (Cap; 0.5 g/liter water) significantly reduced adrenal AT1R binding (maximum binding capacity) by 22% and 19%, respectively, compared with OVX (276 ± 2.09 fmol/mg protein). E2 and Cap lowered adrenal Ang II levels by 39% and 21%, respectively, compared with OVX (4.10 ± 0.44 pmol/g). E2 caused no further reductions in adrenal AT1R binding or in Ang II levels in Cap-treated OVX rats. High-dose Ang II infusion (1000 ng/kg/min) increased adrenal Ang II levels by 71% and lowered AT1R binding by 18%. Under these infusion conditions, E2 did not reduce adrenal Ang II or AT1R binding. No differences in AT1R affinity (dissociation constant) were observed among groups. These data suggest that E2 regulates the number of adrenal AT1R binding sites indirectly by modulating adrenal Ang II.

OVERACTIVATION OF THE renin angiotensin system (RAS) and estrogen deficiency are two of the most highly recognized risk factors implicated in the pathogenesis of cardiovascular diseases (CVD) (1, 2). Medications that antagonize the activity of angiotensin II (Ang II) and aldosterone have clinically beneficial renal and cardiovascular effects (3, 4). Evidence that estrogen is cardioprotective partly comes from the observation that premenopausal women have lower risk for CVD compared with age-matched postmenopausal women, whereas, after menopause, the incidence of CVD dramatically increases (5). Furthermore, estrogen replacement therapy (ERT) has been reported to decrease mean arterial pressure, increase cardiac output, and significantly reduce the risk of developing coronary atherosclerosis, myocardial infarction, and associated CVD in postmenopausal women (6–8). Note that the arm of the Women’s Health Initiative study that was terminated early, due partly to the discovery of increased cardiovascular risk, was the combined estrogen-progestin arm and not the estrogen-alone arm (9, 10).

Whereas 25–50% of the cardiovascular benefits of estrogen have been attributed to the effects of estrogen on the lipid profile (7), most of the protective effects associated with estrogen have not been explained. Some studies have suggested that differential regulation of the RAS by estrogen contributes to the cardiovascular protective benefits associated with estrogen (11, 12). We and others (13) have shown that estrogen alters the expression of angiotensin type 1 receptors (AT1Rs) in many target tissues. We have recently reported that estrogen-decreased adrenal AT1R expression is associated with attenuated adrenal aldosterone responses to Ang II (14). A significant body of knowledge suggests that mineralocorticoid activity is related to the pathogenesis of hypertension and CVD. Rats treated chronically with mineralocorticoids developed hypertension (15). Accompanying features of mineralocorticoid-induced hypertension include several risk factors for cardiovascular events such as increased vascular responsiveness to Ang II, increased vascular resistance, and decreased baroreflex sensitivity. Furthermore, inhibition of aldosterone receptors with the antagonist spironolactone, was recently shown to substantially reduce the risk of morbidity and mortality among patients with severe heart failure who also received standard angiotensin-converting enzyme (ACE) inhibition therapy (5). Thus, we speculate that estrogen-induced decreases in the acute levels of aldosterone secretion may contribute to the cardiovascular benefits associated with estrogen by resulting in decreased overall mineralocorticoid activity.

In this study, we examined the mechanisms involved in estrogen-induced adrenal AT1R reduction. Increasing numbers of studies indicate that estrogen has a regulatory influence on various components of the RAS. Estrogen replacement has been found to increase hepatic production and plasma concentrations of angiotensinogen (16), whereas lowering plasma renin levels and ACE activity (17–22). Ultimately, Ang II synthesis is altered upon estrogen treatment. Because accumulating evidence indicates the existence of a local RAS in various tissues in which the local system serves to limit the actions of Ang II to a specific organ system or physiological event (23), we speculate that, by regulating tissue levels of Ang II synthesis, estrogen decreases adrenal AT1R expression and thereby reduces the control of fluid
homeostasis and blood pressure via modulation of aldosterone secretion from the adrenal gland.

**Materials and Methods**

**Animal treatment**

Female Sprague Dawley rats, weighing approximately 250–300 g, were obtained from Harlan (Indianapolis, IN). All the rats were ovariolectomized (OVX) under isoflurane anesthesia (3% in O2 flowing at 0.5 liters/min). On the next day after surgery, the rats were treated daily with sc injections of 17β-estradiol benzoate (E2) at 40 μg/kg in 0.2 ml peanut oil or vehicle alone. The rats were maintained on a phytoestrogen-free diet (Harlan) and tap water ad libitum under controlled conditions (12-h light, 12-h dark schedule at 24 C). All procedures were approved by the Georgetown University Animal Care and Use Committee.

**Effect of captopril (Cap) treatment on adrenal AT1R expression**

Twelve adult Sprague Dawley rats were subjected to OVX. Cap (Sigma, St. Louis, MO) was dissolved in the drinking water at 0.5 g/liter and infused in the OVX rats at a rate of 200 ng/kg/min through osmotic minipumps (model 2001, ALZET Corp., Palo Alto, CA). Four and 8 d after the treatment, half of the rats within each group (−Cap, n = 6; +Cap, n = 6) were killed by decapitation; the adrenal glands were rapidly excised, then immediately frozen in liquid nitrogen and stored at −80 C.

**Measurement of plasma E2 levels**

Blood (0.5–1 ml) was collected into plain tubes and the serum separated by centrifugation. E2 levels were measured according to the RIA protocol of Diagnostic Products Corp. (Los Angeles, CA).

**Effect of E2 on adrenal AT1R expression and adrenal Ang II levels during Ang II infusion and Cap treatment**

Forty-two rats were OVX and divided into six groups (OVX, n = 7; OVX + E2, n = 7; OVX + Ang II, n = 7; OVX + Ang II + E2, n = 7; OVX + Cap, n = 7; OVX + Cap + E2, n = 7). E2 or peanut oil was injected daily for 7 d. Synthetic Ang II (Sigma) was dissolved in sterile 0.15 m NaCl and infused in the OVX rats at a rate of 200 ng/kg/min through osmotic minipumps (model 2001, ALZET Corp., Palo Alto, CA). Cap was administered through the drinking water at a concentration of 0.5 g/liter in water. After 7 d, the rats were killed, and the adrenal glands were removed for determination of AT1R expression and tissue Ang II levels.

**Effect of E2 on adrenal AT1R expression and adrenal Ang II levels during high-dose Ang II infusion**

Twenty-four rats were OVX and divided into four groups (OVX, n = 6; OVX + E2, n = 6; OVX + Ang II, n = 6; OVX + Ang II + E2, n = 6). E2 or peanut oil was injected daily for 7 d. Synthetic Ang II was dissolved in sterile 0.15 m NaCl and infused in the OVX rats at a rate of 1000 ng/kg/min through osmotic minipumps. Adrenal glands were removed for measurement of AT1R expression and tissue Ang II levels.

**Adrenal AT1R expression**

Membranes from adrenal tissue were prepared and used in radioligand binding assays as previously described (24). Briefly, 0.05 nm [3H]-Sar1, Ile8 Ang II was incubated with 15 μg of adrenal membranes at room temperature for 3 h in binding buffer (100 mmol/liter NaCl; 10 mmol/liter Na2HPO4; 5 mmol/liter EDTA, pH 7.4) supplemented with 0.1% BSA in the presence of the specific AT1R antagonist, PD-123,319 at a 5-μM concentration. Nonspecific binding was determined in the presence of 250 nM nonradioactive Ang II. The AT1R density (maximum binding capacity, Bmax) was determined by Scatchard analysis using the program, GRISMM (GraphPad Software, Inc., San Diego, CA).

**Adrenal Ang II levels**

Ang II was separated from other peptides by HPLC as described before (25, 26). Briefly, adrenals were rapidly weighed and homogenized in chilled 0.18 m HCl/ethanol (1.5 vol/vol). Extracted samples were concentrated on C18 Sep-Pak columns (Waters Associates, Milford, MA), evaporated to dryness, and reconstituted in distilled H2O for HPLC. Recoveries of angiotensin peptides were determined by adding 1000 cpm of 125I-Ang II to the tissues before homogenization.

The peptides were separated on Phenomenex Prodigy ODS reverse-phase column (5 μm, 4.6 × 150 mm) and eluted in a solvent system of 20% acetonitrile in 4 mm triethylammonium formate at a flow rate of 1 ml/min. Fractions were collected from 3–5 min during the run, depending on the retention time of Ang II, and then lyophilized for RIA. The chromatography was conducted in a ThermoSeparation Products P2000 binary pump system with a UV2000 detector at 232 nm, and a Foyo 200 fraction collector. Ang II levels were measured by the Ang II RIA protocol according to American Laboratory Products Co.’s (Windham, NH) product manual.

**Statistical analysis**

The value of each group was averaged and the SEM calculated. Results are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls test for multiple group comparison. Comparisons between two groups were made by using an unpaired t test. P < 0.05 was considered statistically significant.

**Results**

**Effect of Cap on adrenal AT1R expression**

Cap administered in the drinking water at 0.5 g/liter for 4 d significantly decreased the number of adrenal AT1R binding sites (Bmax) by 23% (−Cap, 348 ± 15.3 vs. +Cap, 268 ± 12.5 fmol/mg protein, P < 0.05) (Fig. 1). Eight days of Cap treatment further reduced adrenal AT1R Bmax by 30% (−Cap, 375 ± 28.9 vs. +Cap, 240 ± 5.51 fmol/mg protein, P < 0.01).

**Effect of E2 on adrenal AT1R expression after Cap treatment**

One week of Cap treatment decreased adrenal AT1R Bmax by 19% compared with OVX alone (OVX, 276 ± 2.09 vs. OVX + Cap, 224 ± 14.5 fmol/mg protein, P < 0.05) (Fig. 2). Treatment of OVX animals with E2 (40 μg/kg/d) also de-
creased adrenal AT1R binding; the B_max was reduced by 22% compared with OVX alone (OVX, 2.14 ± 22.1 fmol/mg protein, vs. OVX P < 0.01). In the Cap-treated rats, E2 decreased AT1R B_max by 28% compared with OVX alone but E2 treatment did not further reduce AT1R B_max in the presence of Cap (OVX + Cap + E2, 198 ± 4.84 fmol/mg protein vs. OVX, P < 0.01; vs. OVX + Cap, P > 0.05).

Before surgery, average plasma E2 levels were 13.1 ± 2.7 pg/ml (n = 6). Note that the variability in plasma E2 levels was highest in this group due to summing data from female rats at various stages of their normal estrus cycle; for example, one animal had a plasma E2 level of 2.0 pg/ml, whereas another had a level of 130 pg/ml (27). Seven days after ovariectomy, plasma E2 dropped to 3.4 ± 0.5 pg/ml (n = 10), which is similar to levels typically detected at estrus (27). In the E2-treated OVX rats, plasma E2 was 120 ± 10 pg/ml (n = 11), which represents peak physiological levels reached during the rat estrus cycle (27).

Effect of E2 on adrenal Ang II levels after Cap treatment

Calibration of the HPLC column using synthetic standards of angiotensin peptides revealed a clearly defined UV absorbance sequence of the retention times for Ang I, Ang II, and Ang III (Fig. 3). Angiotensin II was collected between retention times of 3–5 min. The recovery of 125I-Ang II (1000 cpm) added to the adrenal glands before homogenization was 77%.

Cap treatment significantly decreased adrenal Ang II levels in the Cap-treated OVX rats (OVX + Cap + E2, 2.56 ± 0.19 pmol/g tissue vs. OVX + Cap, P > 0.05).

Effect of E2 on adrenal AT1R expression after Ang II infusion at 200 ng/kg/min

Angiotensin II infused at a rate of 200 ng/kg-min (low dose) for 1 wk increased adrenal AT1R B_max in OVX rats by 22% (OVX, 2.14 ± 22.1 fmol/mg protein, vs. OVX P < 0.01) (Fig. 4). As observed previously, 1 wk of E2 treatment (40 µg/kg/d) in OVX rats significantly decreased adrenal AT1R B_max by 22% compared with OVX alone (OVX + E2, 214 ± 22.1 fmol/mg protein vs. OVX, P < 0.01). In the low-dose Ang II-infused rats, E2 treatment also reduced AT1R density (by 30%) compared with the Ang II-infused OVX animals, whereas no significant reduction was detected when compared with OVX alone (OVX + Ang II + E2, 236 ± 9.33 fmol/mg protein vs. OVX + Ang II, P < 0.001; OVX + Ang II + E2 vs. OVX, P > 0.05).

Effect of E2 on adrenal Ang II levels after Ang II infusion at 200 ng/kg-min

Low-dose Ang II infusion (200 ng/kg-min) significantly increased adrenal Ang II levels by 28% compared with OVX alone (OVX, 4.10 ± 0.44 vs. OVX + Ang II, 5.26 ± 0.40 pmol/g tissue, P < 0.05) (Fig. 6). Estradiol treatment in OVX animals decreased adrenal Ang II levels by 39%, as observed above,
and by 17% in the Ang II-infused rats (OVX + E2, 2.51 ± 0.18 pmol/g tissue vs. OVX, P < 0.05; OVX + Ang II + E2, 4.34 ± 0.35 pmol/g tissue vs. OVX + Ang II, P < 0.05).

Effect of E2 on adrenal AT1R expression after Ang II infusion at 1000 ng/kg/min

Angiotensin II infusion at 1000 ng/kg/min markedly reduced AT1R binding sites by 18% (OVX + E2, 168 ± 2.03 fmol/mg protein, P < 0.01) (Fig. 7). As observed previously, E2 treatment in OVX rats significantly decreased AT1R binding sites by 39%; however, no further reductions in AT1R expression were observed in the E2-treated Ang II-infused animals (OVX + E2, 103 ± 6.66 fmol/mg protein vs. OVX, P < 0.001; OVX + Ang II + E2, 135 ± 4.36 fmol/mg protein vs. OVX + Ang II, P > 0.05).

Effect of E2 on adrenal Ang II levels after Ang II infusion at 1000 ng/kg/min

High-dose Ang II infusion (1000 ng/kg/min) significantly increased adrenal Ang II levels by 71% in OVX animals and completely prevented the E2-induced reductions in adrenal Ang II levels (OVX, 4.10 ± 0.44 pmol/g tissue vs. OVX + Ang II, P < 0.01; OVX + Ang II + E2, 7.00 ± 0.52 pmol/g tissue vs. OVX + Ang II, P > 0.05) (Fig. 8).

Discussion

Ang II is well known to regulate its own receptor through both positive and negative feedback mechanisms including Ang II-induced AT1R up-regulation or down-regulation (28–31). Several reports have shown that adrenal AT1R expression is positively regulated by changes in Ang II levels. Dietary sodium restriction in rats has been shown to increase plasma levels of Ang II (32, 33) and under these conditions, adrenal AT1R mRNA and AT1R binding sites are found to be increased by 172% and 161%, respectively (34). Continuous infusion of Ang II at supressor doses (40–200 ng/kg/min) to rats for 1 wk caused a 2-fold increase in adrenal AT1R expression.
mRNA and a 30% increase in AT1R binding sites in the adrenal glomerulosa (35, 36). The ACE inhibitor, lisinopril, reduced adrenal cortex and medulla AT1R binding sites by 33% and 54%, respectively, after 6 wk of treatment (37).

In this study, we show that, under conditions in which Cap treatment inhibited Ang II levels in the OVX rat adrenal tissue by 21%, the number of AT1R binding sites was inhibited to a significant and similar extent (by 19%). Furthermore, we show that subpressor doses of Ang II infused into OVX rats caused a significant increase in adrenal Ang II levels (by 28%) and also similarly increased AT1R binding sites (by 22%). High-dose Ang II infusion markedly increased adrenal Ang II levels by 71%; however, AT1R binding was reduced (by 18%) most likely by ligand-mediated receptor down-regulation. Ang II has been shown to bind to the AT1R to induce receptor-mediated endocytosis and studies suggest that the Ang II-receptor complex undergoes endosomal processing with receptor recycling to the cell surface (38, 39). Although the significance of Ang II-receptor internalization remains unclear, it has been suggested that in the adrenal corticetal, receptor internalization is important in maintaining sustained intracellular signaling after hormonal stimulation (38). Taken together, our current study supports the previous findings indicating that Ang II modulates the expression of its own receptor in a biphasic manner; that is, at low doses, Ang II up-regulates the AT1R, whereas at high doses, Ang II reduces AT1R binding sites by inducing ligand-mediated receptor internalization.

We, and others (40) have previously shown that estrogen regulates AT1R expression. The AT1R density in the rat pituitary has been found to fluctuate with the stage of the estrous cycle, with the highest binding levels found in diestrus (lowest physiological estrogen levels) and the lowest binding found in estrus (highest physiological estrogen levels). In OVX rats, estrogen treatment significantly decreased AT1R number in the pituitary and adrenal by 57% and 67%, whereas in the uterus, estrogen treatment significantly enhanced receptor expression by 180% (13). One mechanism by which estrogen could regulate AT1R expression is through modulating Ang II levels. Brosnihan et al. (22) showed that estrogen treatment decreased ACE activity in both plasma and tissue (aorta and kidney). They also showed that estrogen reduced circulating Ang II levels (22). In this study, we examined the effect of E2 on adrenal levels of Ang II because numerous studies indicate that, in addition to the circulating RAS, many tissues contain local tissue RAS (23). Components of the RAS, such as renin, angiotensinogen, and ACE, are expressed in multiple tissues involved in blood pressure regulation and have shown to be regulated independently of the circulating RAS. In the study of renovascular hypertension, plasma levels of Ang II were found to remain normal despite the persistence of hypertension. In contrast, kidney and adrenal tissue levels of Ang II were markedly up-regulated (41, 42).

All components of the RAS have been identified in the adrenal cortex including renin, angiotensinogen, ACE, and Ang II (43). Renin, angiotensinogen, and ACE are all primarily found in the zona glomerulosa (44–46). The adrenal RAS has been implicated in the regulation of zona glomerulosa tissue growth and function, and in mediating aldosterone release in response to Ang II. Studies using bisected whole adrenal gland showed that Cap inhibited the response of aldosterone to exogenous Ang II stimulation by preventing the conversion of aldosterone from its precursor. These data suggested that the adrenal could regulate Ang II-stimulated aldosterone synthesis and release through the local RAS, independently of the circulating RAS (47). Other studies further demonstrated the dissociation between the circulating RAS and the adrenal-specific RAS in regulating aldosterone production. In the streptozotocin-induced diabetic rat, elevated adrenal aldosterone levels were accompanied by increased adrenal renin activity, even though circulating levels of Ang II were decreased (48). These findings indicate the importance of the adrenal-specific RAS in regulating the physiological and pathophysiological actions of Ang II in the adrenal gland. Thus, our data showing that adrenal levels of Ang II are markedly reduced under conditions in which E2 significantly reduces adrenal AT1Rs and attenuates Ang II-induced aldosterone release supports these previous findings; however, the exact mechanisms remain unclear. E2 could reduce adrenal Ang II levels by inhibiting Ang II synthesis (via lowering local ACE activity) (17, 18) or by preventing adrenal uptake of Ang II from the circulating system through receptor-mediated activities (39).

Several cis-acting regulatory elements including an estrogen response element have been identified in the 5’ flanking region of the AT1R gene, suggesting that estrogen could regulate receptor expression by manipulating AT1R gene transcription (49–52). However, the fact that we did not observe any effect of E2 on adrenal AT1R expression in the Cap-treated rats or in the high-dose Ang II-infused animals, indicates that modulation of adrenal Ang II levels is a necessary component in control of AT1R gene expression because E2 did not modulate adrenal Ang II levels under these conditions. Furthermore, under low-dose Ang II infusion, both adrenal AT1R expression and adrenal Ang II levels were inhibited by E2 treatment.

In contrast to the observed increase in adrenal AT1R binding observed during low-dose Ang II infusion, high-dose infusion decreased adrenal AT1R expression. These observations support previous paradoxical studies showing that Ang II can both up- and down-regulate adrenal AT1 receptors. Under dietary sodium restriction and conditions in which plasma Ang II levels are elevated, adrenal glomerulosa AT1 receptors were up-regulated (53). However, studies on adrenal glomerulosa cells in culture show that Ang II inhibits AT1R expression by inducing ligand-mediated receptor internalization and perhaps reduced recycling to the membrane surface (38, 54, 55). Furthermore, high-dose Ang II infusion in rats markedly decreased kidney glomerular AT1R expression (56, 57). Taken together, these studies suggest that, at low Ang II concentrations, Ang II up-regulates adrenal AT1Rs, whereas at high dose, mechanisms of receptor down-regulation come into play.

We reported previously that estrogen regulates AT1R expression at the posttranscriptional level potentially via cytosolic proteins that bind to cis elements in the 5’ leader sequence of the AT1R mRNA (13). Ang II has also been reported to induce the expression of a variety of inducible transcription factors including the AP-1 family (58). It is thus
possible that estrogen acts through Ang II to regulate transcription factors that mediate the activity of 5' leader sequence mRNA binding proteins; however, it remains unclear whether or not these two mechanisms work independently or coordinately to regulate adrenal AT1R expression.

In conclusion, our study indicates that modulation of adrenal Ang II levels is a necessary component of the molecular mechanisms by which estrogen regulates adrenal AT1R expression. Recent controversy regarding the cardiovascular benefits of estrogen that has arisen from the results of the Women's Health Initiative trial (9, 10) makes determining the mechanisms of estrogen action all the more important in our search to understand the role of estrogen in the progression and incidence of cardiovascular disease.

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