

# In Vivo Investigation of Estrogen Regulation of Adrenal and Renal Angiotensin (AT<sub>1</sub>) Receptor Expression by PET

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The renin angiotensin system (RAS) has been implicated as one mediator of the cardiovascular effects of estrogen. Since changes in angiotensin type 1 (AT<sub>1</sub>) receptor expression are central to modulation of the RAS, we used the noninvasive PET imaging technique to study for the in vivo effects of estrogen on membrane and intracellular AT<sub>1</sub> receptors. **Methods:** Dynamic PET measurements of canine AT<sub>1</sub> (cAT<sub>1</sub>) receptors using the radiolabeled AT<sub>1</sub> receptor antagonist, <sup>11</sup>C-L-159,884, were performed during 2-wk consecutive periods of estrogen deprivation induced by ovariectomy and 17 $\beta$ -estradiol (E<sub>2</sub>) replacement. **Results:** Kinetic modeling of time-activity curves in the kidney and adrenal showed lower receptor expression in the estrogen replete state (21% and 30% decrease in Gjedde-Patlak slope, influx constant, respectively). These in vivo findings correlated with in vitro radioligand-binding assays with [<sup>125</sup>I]-[Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II showing reduced AT<sub>1</sub> receptor number in the adrenal (35%), glomeruli (30%), myocardium (35%), and liver (21%) in the estrogen-replenished compared with estrogen-depleted animals. **Conclusion:** Although other endogenous systems are known to regulate AT<sub>1</sub> receptors and could compete with estrogenic actions, these PET studies reveal that estrogen attenuates AT<sub>1</sub> receptor expression in vivo. Thus, estrogen modulation of AT<sub>1</sub> receptors may contribute to the cardiovascular protective effects associated with estrogen.

**Key Words:** estrogen; renin angiotensin system; PET; AT<sub>1</sub> receptor; angiotensin receptor; estrogen; hormone replacement therapy; adrenal; kidney; heart; liver; regulation; expression

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**E**xperimental research in rats suggests that estrogen administration inhibits the activity of the renin angiotensin system (RAS). Estradiol (E<sub>2</sub>) treatment lowers tissue levels of angiotensin II (Ang II) (1), most likely by its inhibitory action on angiotensin-converting enzyme (ACE) (2), which

converts the inactive decapeptide, Ang I, into the active hormone, Ang II. Ang II is the key mediator of the metabolic and cardiovascular actions of the RAS and mediates its actions by binding to angiotensin type 1 (AT<sub>1</sub>) receptors in various target tissues in animals and humans (3). E<sub>2</sub> attenuates the tissue responsiveness to Ang II action (4), most likely by its inhibitory effects on the number of AT<sub>1</sub> receptors in target tissues (5). E<sub>2</sub> treatment also augments the vasodilator effects of angiotensin<sub>1-7</sub> (6).

So far, investigation of the interaction between estrogen and the RAS in humans has been limited to assessing changes in the circulating RAS components (7,8). These studies show that estrogen administration leads to an increase in plasma levels of angiotensinogen and plasma renin activity (PRA) (7), indicating that estrogen activates some components of the human RAS cascade. However, estrogen treatment also produced a reduction in ACE (8). These studies suggest that, even though some of the upstream components of the RAS cascade are activated, the overall effect of estrogen is to attenuate the activity of the AT<sub>1</sub> receptor, the key effector of Ang II action in its target tissues. Until now, the effect of estrogen on AT<sub>1</sub> receptor expression in humans (hAT<sub>1</sub>) or any other species that has only one known functional subtype of the AT<sub>1</sub> receptor has not been reported.

In this study, we examined the effect of estrogen on canine AT<sub>1</sub> (cAT<sub>1</sub>) receptor expression since the cAT<sub>1</sub> and hAT<sub>1</sub> receptors share many similar structural and regulatory elements (9,10). We used the noninvasive PET imaging technique to study the effects of estrogen on membrane and intracellular AT<sub>1</sub> receptors in an in vivo setting that has the advantage of encompassing other endogenous systems that may regulate AT<sub>1</sub> receptor action (11,12). PET measurements of cAT<sub>1</sub> receptor expression in the kidney and adrenal of adult female beagles were performed during periods of estrogen deprivation and estrogen replacement in the same animal. At the end of the in vivo studies, organs were harvested and AT<sub>1</sub> receptor numbers in target tissues, including the kidney, adrenal, heart, and liver, were deter-

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mined by an in vitro radioligand-binding assay. Levels of circulating components of the RAS as well as serum and urine electrolytes were also measured under these conditions.

## MATERIALS AND METHODS

### Animal Protocol

The Animal Care and Use Committee of The Johns Hopkins Medical Institutions and Georgetown University approved the experimental guideline and protocol. Eight adult premenopausal female beagles (average weight, 11.6 kg) were used in the study. The animals were allowed an initial period of 1 wk to acclimatize to the animal holding area. They were fed a special standardized diet (H/D diet; Hill's Pet Nutrition) enriched with normal daily salt intake (60 mEq/d total NaCl) and allowed water intake ad libitum throughout the duration of the study.

After 1 wk of acclimation, bilateral oophorectomy was performed on all dogs. Briefly, after premedication with acepromazine (0.03–0.05 mg/kg), anesthesia was induced with thiopental (10–20 mg/kg) and maintained with isoflurane. After standard skin preparation, an abdominal midline surgical incision was made. Using the uterus as a guide, the ovaries were identified and exteriorized. The vascular supply was identified, laid open, and ligated in between clamps, after which the 2 ovaries along with the ligaments were delivered in 1 piece. Complete removal of the ovaries and uterus was confirmed by ex situ serial sectioning and visual inspection. The incisions were closed in a routine fashion and the procedures were well tolerated by all animals.

While observing aseptic precautions, a slow-release E<sub>2</sub> pellet (17 $\beta$ -estradiol, 25 mg per pellet, 21-d slow release; Innovative Research of America) was introduced into the subcutis through a stab incision placed over the right inferior border of the rib cage. To facilitate future removal, the pellet was maneuvered to sit directly on the rib before closing the skin.

After oophorectomy, the dogs were divided into 2 treatment groups. One group had immediate E<sub>2</sub> replenishment lasting for 2 wk followed by another 2 wk without E<sub>2</sub> (on–off model). The second group remained E<sub>2</sub> deficient for the initial 2 wk after surgery and was thereafter placed on E<sub>2</sub> replenishment for 2 wk (off–on model). In each animal, renal and adrenal AT<sub>1</sub> receptor levels were assessed in vivo with PET imaging after 2-wk periods of E<sub>2</sub> replacement as well as after 2-wk periods of E<sub>2</sub> deficiency.

### Imaging and Image Analysis

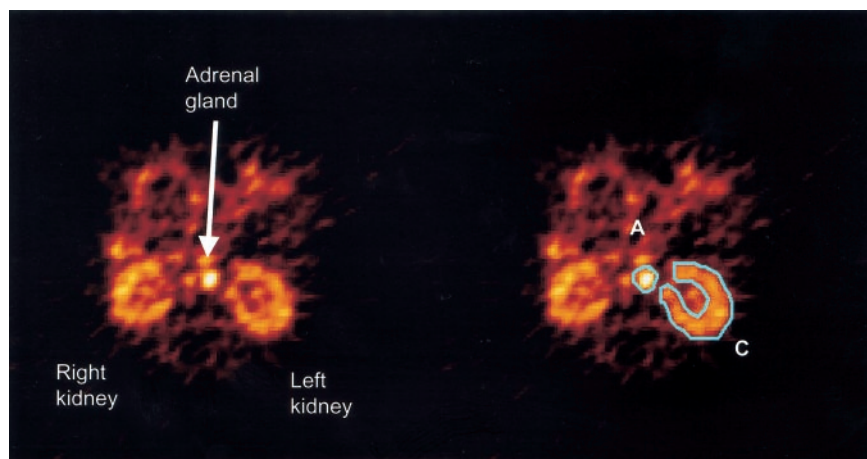
Animal preparation for PET involved fasting for at least 12 h before imaging but free water intake was allowed. Serum and plasma samples were collected before PET study for estimation of electrolytes (Na, K, Cl), blood urea nitrogen (BUN), creatinine, aldosterone, and PRA.

The radioligand <sup>11</sup>C-L-159,884 (*N*-[[4'-(2-ethyl-5,7-dimethyl-<sup>3</sup>H-imidazo[4,5-*B*]pyridine-3-yl)methyl][1,1'-biphenyl]-2-yl]sulfonyl]-4-methoxybenzamide) was synthesized according to Hamill et al. (13). PET studies were performed in animals under pentobarbital anesthesia with a General Electric 4096+ (GE Medical Systems) as described previously (14). The in-plane-cross-plane resolution of this scanner is 6-mm full width at half maximum. The average injected dose was 552  $\pm$  32 MBq (14.9  $\pm$  0.9 mCi) at an average specific activity of 71  $\pm$  40 GBq/ $\mu$ mol (2,206  $\pm$  836 mCi/ $\mu$ mol). After performing a transmission scan with a 370-MBq (10 mCi) <sup>68</sup>Ge pin source, the radioligand was injected intravenously as a slow bolus of 5- to 10-s duration and the following image sequence of the mid abdomen was acquired: four 15-s frames, three 1-min frames, three 5-min frames, three 10-min frames, and one 20-min frame. Since the images were reconstructed with a ramp filter, the spatial resolution of the PET scans was 6 mm.

To obtain tissue activity curves, regions of interest (ROIs) were defined to include the left renal cortex and left adrenal, which was shown to generate more reproducible results than the right side where partial-volume effects from the liver are significant (15).

Pixels with >30% of maximal renal cortical (or adrenal) activity were included in the ROIs. The obtained time–activity curves were corrected for radioisotope decay and were expressed in Bq/mL/MBq (nCi/mL/mCi) injected dose. ROIs were defined on all slices showing the adrenal and the kidney. The kidney cortex was easily identifiable and separable from the medulla. In contrast, it was impractical to separate the adrenal cortex from the adrenal medulla. Thus, the kidney ROI included only the cortex, whereas the adrenal ROI included both the cortex and medulla. Therefore, tissue radioactivity concentration was analyzed in the renal cortex, whereas total tissue activity was analyzed in the entire adrenal gland (Fig. 1).

Plasma radioactivity was measured in 0.3 mL arterial blood collected every 5–8 s during the first 2 min after injection and at increasing time intervals thereafter. Five additional samples (2 mL) were collected at 5, 15, 30, 60, and 90 min after injection for



**FIGURE 1.** Accumulation of <sup>11</sup>C-L-159,884 in kidney and adrenal 55–95 min after injection (left image) and ROIs of left kidney and left adrenal gland (right image). ROIs were defined on all image slices showing organs. ROI of kidney included only renal cortex (C), whereas ROI of adrenal included entire gland (A).

determination of radioligand metabolites by high-performance liquid chromatography (HPLC). Plasma radioactivity was cross-calibrated with the PET scans, corrected for decay and expressed in Bq/mL/MBq (nCi/mL/mCi) injected dose. The plasma curve was also corrected for metabolites by multiplying with the unmetabolized fraction obtained by HPLC. Since the average number of data points for the uncorrected input function was 35 compared with only 5 data points for HPLC, the percentage of unmetabolized ligand at the 35 time points was calculated by biexponential curve fitting of the 5 HPLC measured values.

This was different from the previous study (14) in which the missing points of the unmetabolized tracer were estimated by a monoexponential fit. Not only the addition of 1 more sample but also the significantly lower variance of the measurements with the recently published new technique (16) contributed to this more accurate fit.

Organ uptake of  $^{11}\text{C}$ -L-159,884 was quantified by graphical (Gjedde-Patlak) analysis (14) using the last 6 data points of the graphical plot. The radioligand influx constant  $K_i$  was used as outcome measure. The measurements were not corrected for tissue density; therefore,  $K_i$  was expressed in units of mL/min/mL.

### In Vitro Measurements

PRA was determined by radioimmunoassay (Angiotensin I [ $^{125}\text{I}$ ] RIA Kit; Perkin Elmer) on 100- $\mu\text{L}$  plasma samples in quadruplicate from blood collected in the presence of ethylenediaminetetraacetic acid (EDTA) (8.55 mg  $\text{K}_3\text{EDTA}$ ), at days 0, 15, and 30. The standard range of the assay was 0.1–10 ng/mL; the PRA was expressed as ng/mL/h of generated Angiotensin I. Quality control of the assay was ensured by using 3 serum controls (Lyphochek Hypertension Markers Control; Bio-Rad).

Aldosterone was determined by radioimmunoassay (Coat-A-Count Aldosterone; Diagnostic Product Corp.) on 200- $\mu\text{L}$  plasma samples in duplicate from blood collected in the presence of heparin (100 U heparin). The standard range of the assay was 25–1,200 pg/mL. Quality control of the assay was ensured by using 3 serum controls (Multivalent Control Module; Diagnostic Product Corp.).

On day 30, the animals were killed and the tissues were removed to quantify  $\text{AT}_1$  receptor number by radioligand-binding assay. The glomeruli, adrenal glands, hearts, and livers from each dog were homogenized and membranes were isolated as previously described (12,17).  $\text{AT}_1$  receptor numbers were determined in radioligand saturation experiments using  $^{125}\text{I}$ -[Sar<sup>1</sup>,Ile<sup>8</sup>]Ang II as the radiolabeled ligand. Radioactivity was measured in a Beckman

$\gamma$ -counter. The maximum number of binding sites ( $B_{\text{max}}$ ) was calculated from Scatchard plots using the program PRISM (Graph-Pad Software Inc.) and was expressed as fmol/mg of protein (12).

### Statistical Analysis

Results were expressed as mean  $\pm$  SEM. Since identical animals were used for the on and off  $\text{E}_2$  measurements, differences in  $K_i$  values were tested using the paired unequal variance,  $t$  test. A regular Student  $t$  test was used to analyze  $B_{\text{max}}$ , PRA, and aldosterone changes in response to ovariectomy and  $\text{E}_2$  treatment. For both types of tests,  $P < 0.05$  was considered statistically significant. No corrections were applied for testing differences of multiple parameters. Due to lack of a normal distribution, the plasma levels of  $\text{E}_2$  and aldosterone were compared using the nonparametric Kruskal–Wallis test.

### RESULTS

Arterial blood pressure monitoring showed that  $\text{E}_2$  depletion caused a marginal and statistically insignificant increase in average systolic and diastolic blood pressure from  $130/74 \pm 6/3$  mm Hg on  $\text{E}_2$  to  $138/77 \pm 6/3$  mm Hg off  $\text{E}_2$ .

Serum levels of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{HCO}_3^-$ , BUN, and creatinine measured in all animals were within the normal reference range during both phases of treatment (Table 1). Similarly, estimation of urine electrolytes and osmolality revealed values within the normal reference range (Table 1). Nonetheless, reduction in urinary excretion of  $\text{Na}^+$ ,  $\text{K}^+$ , and urine osmolality with  $\text{E}_2$  administration was observed; however, due to a large variance of these parameters, only the differences in urine osmolality were statistically significant.

PRA and plasma levels of  $\text{E}_2$  and aldosterone were assayed before each PET study.  $\text{E}_2$  levels were below the limits of assay detection in animals off  $\text{E}_2$ , whereas those on  $\text{E}_2$  showed a range of 241–425 pg/mL (average,  $318 \pm 34$  pg/mL;  $P < 0.05$ ). The average PRA was 1.63 ng/mL/h in animals deprived of  $\text{E}_2$ , which increased to 1.85 ng/mL/h during the  $\text{E}_2$ -replenished phase ( $P < 0.05$ ). Average plasma aldosterone levels were  $26 \pm 11$  pg/mL in the hormone-deprived animals but were below the limits of assay detection in the  $\text{E}_2$ -replenished animals (Table 2) ( $P < 0.05$ ). Two dogs, one from each protocol, were withdrawn from this analysis because the baseline levels of

**TABLE 1**  
Vital Signs, Urine, and Serum Electrolytes During Estrogen-Deficient and Estrogen-Replete Phases of Study

Phase	Parameter											Urine osmolality (mOsm/kg $\text{H}_2\text{O}$ )
	HR (beats/min)	SBP (mm Hg)	DBP (mm Hg)	Serum Na (mEq/L)	Serum K (mEq/L)	Serum Cl (mEq/L)	Serum $\text{HCO}_3^-$ (mEq/L)	BUN (mg/dL)	Creatinine (mg/dL)	Urine Na (mEq/L)	Urine K (mEq/L)	
Off estrogen	$103 \pm 7$	$138 \pm 6$	$77 \pm 3$	$148 \pm 2$	$4.0 \pm 0.6$	$115.0$	$13.0 \pm 3$	$5.0 \pm 1$	$0.5 \pm 0.1$	$104 \pm 38$	$81.2 \pm 18$	$994 \pm 265$
On estrogen	$101 \pm 4$	$130 \pm 6$	$74 \pm 3$	$154 \pm 4$	$3.5 \pm 0.2$	$123 \pm 4$	$22.5 \pm 2$	$3.5 \pm 2$	$0.5 \pm 0.1$	$29.0 \pm 17$	$32 \pm 16$	$240 \pm 133^*$

\* $P < 0.05$ .

HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure.

Data are expressed as mean  $\pm$  SEM.

**TABLE 2**  
Circulating and Tissue RAS Components During Both Phases of Study, as Measured In Vivo and In Vitro

Phase	E <sub>2</sub> (pg/mL)	PRA (ng/ mL/h)	Aldosterone (pg/mL)	AT <sub>1</sub> R in vivo (K <sub>i</sub> )		AT <sub>1</sub> R B <sub>max</sub> in vitro (fmol/mg protein)			
				Renal	Adrenal	Glomerular	Adrenal	Liver	Myocardial
Off									
estrogen	ND	1.37 ± 0.54	26 ± 11	0.022 ± 0.006	0.036 ± 0.011	1,243 ± 56	253.4 ± 20	70.7 ± 3	16.8 ± 1
On									
estrogen	317.9 ± 96*	1.85 ± 0.5*	ND*	0.017 ± 0.005*	0.025 ± 0.007*	873.3 ± 42*	165.9 ± 12*	55.9 ± 3*	11.1 ± 2

\*P < 0.05.  
AT<sub>1</sub>R = AT<sub>1</sub> receptor; ND = not detectable.  
Data are expressed as mean ± SEM.

aldosterone were outside of the baseline level interquartile range and results were significantly different from reported values in dogs using the same assay method (18).

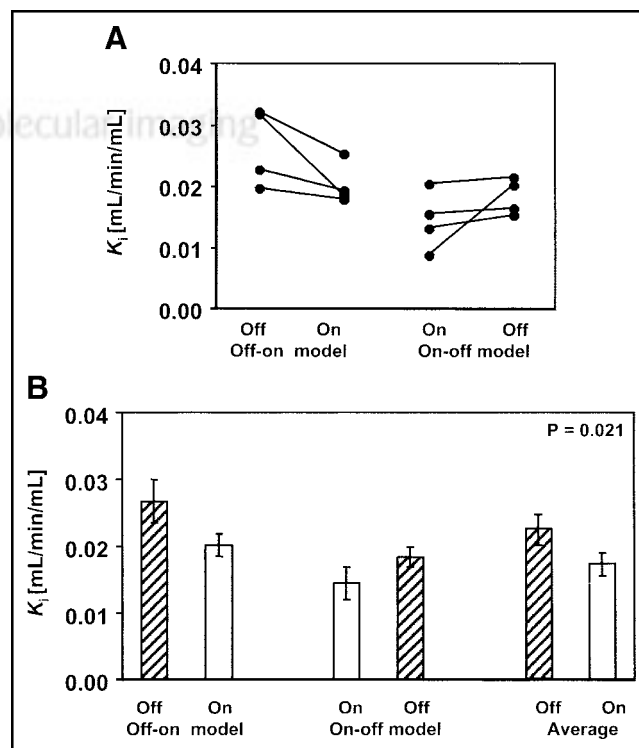
There was a significant change in AT<sub>1</sub> receptor expression with modulation of the hormonal state. E<sub>2</sub> administration caused declines of 21% and 30% in renal and adrenal Gjedde–Patlak slopes (K<sub>i</sub>), respectively (Table 2; Figs. 2 and 3). In vitro radioligand assays showed reductions of 30% and 35% in AT<sub>1</sub> B<sub>max</sub> in kidney glomeruli and adrenal gland, respectively (Table 2; Fig. 4). Although the differences were similar, there was no quantifiable linear correlation between B<sub>max</sub> and K<sub>i</sub>.

Animals in the on–off model showed 25% increase, whereas animals in the off–on model showed 32% decrease in renal K<sub>i</sub> (Fig. 2). The change in adrenal K<sub>i</sub> was similar: 31% increase for the on–off group and 35% decrease for the off–on group (Fig. 3). These changes could not be attributed to changes in the input function. The average unmetabolized <sup>11</sup>C (i.e., the parent compound fraction) was 57.1% ± 2.8% in the animals on and 58.7% ± 2.2% in the animals off E<sub>2</sub>. The differences in the parent compound fraction were not statistically significant (paired *t* test *P* = 0.630). On the other hand, the in vivo measurements were confirmed by in vitro data. Two weeks of E<sub>2</sub> treatment resulted in significant decreases in AT<sub>1</sub> receptor B<sub>max</sub> determined in vitro, in membranes prepared from the adrenal (35%, *P* < 0.05), liver (21%; *P* < 0.05), myocardium (35%), and isolated glomeruli (30%; *P* < 0.05), compared with estrogen-depleted animals (Table 2; Fig. 4). B<sub>max</sub> was 4 times higher in the liver than in the myocardium (Table 2).

## DISCUSSION

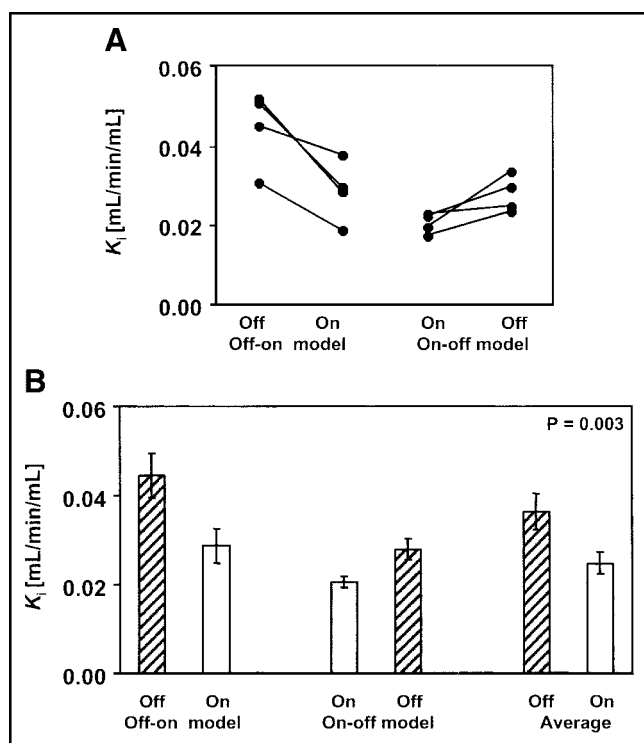
PET imaging revealed that E<sub>2</sub> replacement after ovariectomy led to lower in vivo AT<sub>1</sub> receptor radioligand binding in the renal cortex and adrenal compared with their E<sub>2</sub>-depleted state. This finding agrees with reports from in vitro studies by us (12) and by other investigators, which showed that E<sub>2</sub> administration causes a downregulation of AT<sub>1</sub> receptor expression in diverse tissues, including the hypothalamus, pituitary (5,19), adrenal gland (5), and aortic vascular

wall (4). To our knowledge, this is the first study of estrogen action in a species that, like humans, has only one known functional subtype of the AT<sub>1</sub> receptor. Furthermore, this is the first time estrogen action on AT<sub>1</sub> receptors has been examined in vivo. Dogs subjected to hormonal deprivation and then E<sub>2</sub> replenishment exhibited a 32% and 35% overall reduction in renal and adrenal AT<sub>1</sub> receptor binding, respec-



**FIGURE 2.** Effect of estrogen depletion on cAT<sub>1</sub> receptor expression in renal cortex as determined by PET imaging in vivo. (A) K<sub>i</sub> changes in individual animals first off and then on E<sub>2</sub> (off–on model) and in animals first on and then off E<sub>2</sub> (on–off model). (B) K<sub>i</sub> in 2 groups of animals (off–on and on–off model) and in all pooled animals off and on E<sub>2</sub>. Differences between off and on E<sub>2</sub> were consistent but statistically not significant in separate off–on and on–off subgroups. Differences between pooled parameters were statistically significant (*P* = 0.021).





**FIGURE 3.** Effect of estrogen depletion on cAT<sub>1</sub> receptor expression in adrenal gland as determined by PET imaging in vivo. (A)  $K_i$  changes in individual animals first off and then on E<sub>2</sub> (off-on model) and in animals first on and then off E<sub>2</sub> (on-off model). (B)  $K_i$  in 2 groups of animals (off-on and on-off model) and in all pooled animals off and on E<sub>2</sub>. As in kidney (Fig. 2), differences between off and on E<sub>2</sub> were consistent but statistically not significant in separate off-on and on-off subgroups. Differences between pooled parameters were statistically significant ( $P = 0.003$ ).

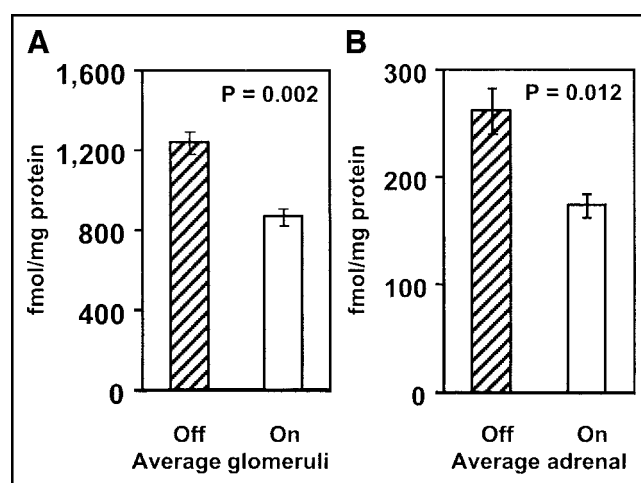
tively, compared with their E<sub>2</sub>-deficient state (Table 2). Using the Gjedde-Patlak plot for quantification of radioligand binding, all 8 dogs studied showed this down-regulatory effect of E<sub>2</sub> on cAT<sub>1</sub> receptor expression in the kidney and adrenal gland.

The  $K_i$  parameter depends not only on receptor binding of the radioligand but also on uptake and release in the kidney (20). In this specific set of experiments, the observed changes of  $K_i$  can be applied to measure AT<sub>1</sub> receptor regulation for the following reasons: (a) The contribution of specific binding to the total variance of  $K_i$  is 67% (14). (b) Dietary sodium, a strong regulator of the AT<sub>1</sub> receptor, does not affect renal blood flow measured with PET (15). (c) E<sub>2</sub> has been shown to increase renal blood flow only by 12% (21) but such a change would have had an effect on the AT<sub>1</sub> receptor opposite to those observed in the present experiments. (d) It is unlikely that metabolites of the radioligand, <sup>11</sup>C-L-159,884, contributed to  $K_i$  since the amount of metabolized radioligand is <15% of the total <sup>11</sup>C concentration in the renal parenchyma. This fraction remains stable with time after radioligand injection and is independent of the regulatory changes of the AT<sub>1</sub> receptor (22). Also, the

fraction of metabolized ligand in the plasma of animals on or off E<sub>2</sub> was comparable. (e) Most importantly, in vivo differences in  $K_i$  are supported by differences of  $B_{max}$  determined in vitro (Table 2; Figs. 2–4).

The renal and adrenal cAT<sub>1</sub> receptor expression is coordinately regulated and strongly influenced by dietary sodium intake (10). This is a confounding variable that we had to guide against by ensuring that all animals were placed on a standardized diet enriched with normal daily salt intake. In addition, we determined the serum and urine electrolytes and urine osmolality before each PET study, and all measured values were within the reference range. Although increased urinary sodium reabsorption was noted in the presence of E<sub>2</sub>, this did not produce severe alterations in electrolytes since individual values were within the normal reference range and there was no significant difference in the average serum and urine electrolyte levels between the 2 treatment groups (Table 1). There were, however, significant differences in urine osmolality between the off-on and on-off phase, perhaps, as a consequence of the increased sodium reabsorption induced by E<sub>2</sub>. This finding is not surprising since E<sub>2</sub> causes increased renal reabsorption of fluid and sodium independent of aldosterone levels (23). This effect of E<sub>2</sub> is probably due to the hormone's direct effect on mineralocorticoid activity (24) and possibly, also, through its action on the expression and localization of a NaCl cotransporter in the distal convoluted tubules (25,26).

We observed a 27% increase in PRA in the E<sub>2</sub> repletion phase of the study compared with the E<sub>2</sub> deprivation phase (Table 2). However, there was no associated increase in plasma aldosterone levels with the raised PRA. In fact, plasma aldosterone levels were below the detection limit of our assay method during the E<sub>2</sub> replete phase. This seeming discordance between PRA and aldosterone levels may be explained by the other known actions of E<sub>2</sub>, including



**FIGURE 4.** Effect of estrogen on cAT<sub>1</sub> receptor expression as measured by in vitro radioligand binding expressed as  $B_{max}$  in units of fmol/mg protein. Differences were significant in both glomerular (A) ( $P = 0.002$ ) and adrenal (B) ( $P = 0.012$ )  $B_{max}$ .

reduced ACE messenger RNA (2), reduced tissue sensitivity to Ang II (4,5), and, especially, reduced expression of the AT<sub>1</sub> receptor in the adrenal (5). Furthermore, E<sub>2</sub> administration acts centrally to suppress pituitary AT<sub>1</sub> receptor expression, thereby causing reduced adrenocorticotrophic hormone and aldosterone production (12,19). It is likely that these effects of E<sub>2</sub> counteracted the consequences of increased PRA, thus producing an overall downregulation of the RAS. Although several investigators have also reported increased PRA with E<sub>2</sub> administration (27), Schunkert et al. reported a fall in active renin concentrations in the plasma in postmenopausal women on estrogen replacement therapy (ERT) compared with those not on ERT (7). One possible explanation for the discrepancy between Schunkert's findings and those of other investigators is the assay method used since the traditional indirect PRA assay correlates poorly with the direct monoclonal antibody active renin assay when the PRA level is <2 ng/mL/h (27). Although further studies may be needed to fully elucidate the effect of E<sub>2</sub> on plasma renin, our data suggest that the overall effect of E<sub>2</sub> is to reduce the activity of the RAS by lowering AT<sub>1</sub> receptor expression and, consequently, reduced tissue responsiveness to Ang II. Thus, suppression of the RAS by estrogen could be a contributory mechanism accounting for the cardiovascular health benefits associated with estrogen.

Whereas the in vivo measurements were repeated in the same animal, we could only perform one in vitro assay in each animal. Nonetheless, by having one group of dogs on E<sub>2</sub> and the other group off E<sub>2</sub> at the time of killing, we were able to study the effect of E<sub>2</sub> on AT<sub>1</sub> receptor expression in vitro. This in vitro assessment was also used to validate the in vivo PET measurements. In vitro binding assays revealed a 30% decrease in glomerular B<sub>max</sub> and a 35% decrease in adrenal cAT<sub>1</sub> B<sub>max</sub> in animals on E<sub>2</sub> compared with those off E<sub>2</sub> (Table 2; Fig. 4). This change is consistent with in vivo PET assessments.

The K<sub>i</sub> parameter that was derived from the PET studies depends on the density of receptors (B<sub>max</sub>) in a linear fashion (28); thus, similar relative changes of the AT<sub>1</sub> receptor are expected when assessed by in vitro and in vivo binding experiments if other physiologic parameters, such as renal blood flow and nonspecific binding of the radioligand, remain unchanged.

The presented in vitro assay results are also comparable with our previous data showing a 45% reduction in adrenal rat AT<sub>1</sub> receptor number after E<sub>2</sub> replacement in NaCl-deprived ovariectomized rats (12). The lack of correlation between individual in vitro and in vivo renal AT<sub>1</sub> receptor values can be partly explained by the fact that assays were performed on isolated glomeruli rather than whole kidney preparations. Also, the use of unlabeled AT<sub>1</sub> receptor- and AT<sub>2</sub> receptor-specific ligands for saturation and competition studies made for greater accuracy in estimating the amount of non-AT<sub>1</sub> receptor binding in vitro (15). The in vitro studies also demonstrated downregulation of myocardial AT<sub>1</sub> receptor binding in animals on E<sub>2</sub>, a result that is

of special importance considering the potential cardioprotective effects of ERT. Receptor density (B<sub>max</sub>) was much lower in the myocardium than in the liver, adrenal, or kidney (Table 2). This large difference in the number of binding sites and the fact that the PET radioligand is excreted through the hepatobiliary route may hamper imaging of the myocardium—in particular, imaging of the inferior wall. Similarly, high receptor binding in the liver combined with hepatobiliary excretion can interfere with receptor quantification in the right adrenal and the upper pole of the right kidney.

The RAS regulates blood pressure and fluid homeostasis through AT<sub>1</sub> receptors in various target tissues. Thus, downregulation of AT<sub>1</sub> receptors is likely to reduce the tissue responsiveness to Ang II in these tissues. In the kidney, AT<sub>1</sub> receptors mediate glomerular blood flow and sodium, chloride, and bicarbonate transport processes in the renal tubules (29). In the adrenal gland, stimulation of AT<sub>1</sub> receptors results in aldosterone secretion from the adrenal cortex (30) and increased catecholamine synthesis in the adrenal medulla (31). In the heart, AT<sub>1</sub> receptors mediate vascular contractility as well as growth and hypertrophy (32). Although the effects in the liver are not well understood, AT<sub>1</sub> receptors are known to modulate hepatic metabolism, including glucose and lactate balance (33). Attenuation of all or some of these actions could contribute to the cardioprotective effects associated with estrogen.

## CONCLUSION

This study examines the effects of estrogen on AT<sub>1</sub> receptor expression in vivo. It shows that E<sub>2</sub> regulates the RAS in dogs, causing reduced expression of the cAT<sub>1</sub> receptor in the kidney, adrenal gland, liver, and heart. These receptor changes are reversible and measurable with PET imaging. PET measurement of changes in AT<sub>1</sub> receptor expression is comparable with in vitro assays and may hold great potential for further investigation of the effect of hormone replacement therapy in humans. Clarification of the precise molecular basis of estrogen action is likely to lead to the development of new hormone replacement therapeutics, which possess the optimum benefits of estrogen action while minimizing the adverse effects.

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