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Estrogen upregulates renal angiotensin II AT_1 and AT_2 receptors in the rat

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Abstract

We studied renal AT_1 and AT_2 receptors in male, female, ovariectomized and ovariectomized-estrogen-treated Wistar–Hanover and Wistar–Kyoto rats. AT_1 receptors and AT_{1A} receptor mRNA predominated, with no significant differences between males and females. AT_2 receptor expression was restricted in female rats to the capsule, the transition zone between outer and inner medulla, the endothelium lining the papilla, and arcuate arteries and veins. There were no AT_2 receptors in male rats, while male mice express substantial numbers of estrogen-dependent AT_2 receptors. Arcuate arteries and veins expressed AT_{1B} mRNA in males and females, and AT_2 mRNA in females only. AT_1 receptor and AT_2 receptor expression were estrogen-dependent, with increases in AT_1 and AT_2 receptor expression after estrogen treatment in ovariectomized rats. Estrogen treatment increased prostaglandin E_2 (PGE_2) and cGMP concentrations in the renal medulla, and eNOS expression in cortical arteries. In rodents, expression of renal Angiotensin II receptor types is estrogen-dependent, with significant species, strain and area differences. Our results support an important role for AT_2 receptors in the regulation of renal function and in the protective effects of estrogen in the kidney.
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Keywords: Renin–angiotensin system; Ovarian hormones; Kidney function; Prostaglandins

1. Introduction

Circulating Angiotensin II (Ang II) is produced by the kidney by a local renin–angiotensin system (RAS). Through stimulation of its physiologically active AT_1 receptors, Ang II regulates tubular sodium and water reabsorbtion, renal blood flow, and glomerular filtration rate [1,2]. In the kidney, autoradiography, in situ hybridization and immunocytochemical studies established AT_1 receptors as the predominant Ang II receptor type. These receptors are localized in the adult mammalian kidney predominantly to glomeruli, with lower levels in renal cortical tubules, vasculature, medullary interstitial cells, and collecting ducts [3–6]. Pathological AT_1 receptor stimulation increases vasoconstriction and sodium retention [1,7] and, independently, produces mesangial cell hypertrophy and renal injury [8].

The function of the second Ang II receptor type, the AT_2 receptor, is controversial [9]. In male rats, high expression of renal AT_2 receptors in the fetal kidney suggested a role during kidney development and organogenesis [10]. Conversely, in the adult male rat kidney, autoradiography findings indicated that AT_2 receptors were no longer expressed [10,11], and immunocytochemical studies detected AT_2 receptors only at very low levels [12]. These studies suggested that renal AT2 receptors played, if any, only a minor role in the regulation of adult kidney function.

Studies in other mammalian species, however, indicated the possibility of a major role of AT_2 receptors in the function of the adult kidney. In humans, AT_2 receptor mRNA is localized in blood vessels, tubular structures, and glomeruli [13]. In rabbits, AT_2 receptor stimulation dilates renal afferent arterioles [14]. In adult male mice, AT_2 receptors are clearly expressed and associated with renal blood vessels [4]. These observations indicated a participation of renal AT_2 receptors in the regulation of kidney vascular flow and perhaps other functions [7,14,15]. AT_2 receptor stimulation not only produced renal vasodilation [14], but also decreased mesangial cell hypertrophy [16] and was natriuretic [15]. This suggested that in the kidney, AT_2 receptor stimulation acted in opposition to and in balance with AT_1 receptors, and that their stimulation could have beneficial effects.

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In rodents, most of the earlier studies on renal Ang II receptor types have been performed in males. We have found that female mice expressed much higher numbers of renal AT_2 receptors than those present in male mice, and that the expression of renal AT_2 receptors in female mice was estrogen-dependent [17]. We hypothesized that the presence of high numbers of estrogen-dependent AT_2 receptors in the female kidney, by a modification of the renal AT_1/AT_2 receptor balance [17], could be related to the postulated effects of estrogen replacement therapy, the prevention of development of hypertension, and the delayed progression of renal disease [18,19].

To further clarify the regulatory influence of estrogens on renal Ang II receptors we studied AT_1 and AT_2 receptor expression in male, female, ovariectomized and estrogen-treated ovariectomized Wistar–Hanover and Wistar–Kyoto rats. We studied two different rat strains to determine to what extent any observed difference could be generalized to the species studied or considered to be strain-dependent.

2. Material and methods

2.1. Animals

Groups of six to seven 8-week-old male, female and ovariectomized female Wistar–Hanover and Wistar–Kyoto rats were obtained from Taconic (Germantown, NY) and kept under controlled conditions with free access to water and food, according to protocols approved by the NIMH Animal Care and Use Committee. We compared male, sham-operated females implanted with a cholesterol pellet, ovariectomized rats implanted with a cholesterol pellet and ovariectomized rats treated with estrogen to study the influence of estrogen on renal Ang II receptor type and eNOS expression and PGE_2 and cGMP levels in inner medulla containing the transition zone. Females were sacrificed during the diestrous I stage of their cycle. Fourteen days after ovariectomized or sham operation, female rats were implanted subcutaneously with 17β-estradiol or cholesterol placebo pellets (1.7 mg/pellet–60 days release; Innovative Research of America, Sarasota, FL) providing about 28 μg 17β-estradiol per rat per day, with a blood level of 180 ± 25.5 pg/ml under pentobarbital anesthesia (30 mg/kg, i.p.). Ten days after the pellets were implanted, all animals were killed by decapitation between 10:00 AM and 11:00 AM and the kidneys were immediately removed, frozen at −30 °C by immersion in isopentane kept on dry ice at −30 °C and stored at −80 °C.

2.2. Quantitative autoradiography of Angiotensin II receptor types

For binding studies, consecutive 16-μm-thick kidney sections were cut in a cryostat at −20 °C, thaw-mounted on polylysine-coated slides (Polysine, Erie Scientific, Portsmarth, NH), dried overnight in a desiccator at 4 °C and kept at −80 °C until used. Sections were preincubated for 15 min at 22 °C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM EDTA, 0.005% bacitracin (Sigma, St. Louis, MO), and 0.2% protease-free BSA (Sigma), followed by incubation for 120 min at 22 °C in fresh buffer, prepared as above with the addition of 0.5×10^{-9} mol/l [^{125}I]Sarcosine^1–Angiotensin II ([^{125}I]Sar^1–Ang II) from Peninsula Laboratories (Belmont, CA), iodinated by the Peptide Radioiodination Service Center, Washington State University (Pullman, WA) to a specific activity of 2176 Ci/mmol, to determine total binding. Binding of [^{125}I] Sar^1–Ang II to AT_1 receptors was determined in adjacent kidney sections incubated as above with the addition of 10^{-5} M losartan (DuPont-Merck, Wilmington, DE), to selectively displace binding to AT_1 receptors [20]. Binding to AT_1 receptors was the difference between total binding and the binding remaining in adjacent sections incubated in the presence of an excess concentration of losartan.

Binding of [^{125}I]CGP-42112 iodinated by the Peptide Radioiodination Service Center, specific activity 2176 Ci/mmol, Washington State University (Pullman, WA) was performed in another set of adjacent sections to determine the specific binding to AT_2 receptors. Sections were preincubated as above followed by incubation for 120 min in fresh buffer containing 0.2×10^{-9} mol/l [^{125}I]CGP-42112. Nonspecific binding was determined by incubating consecutive sections with 5×10^{-6} mol/l unlabeled Ang II (Peninsula). At the concentrations used, [^{125}I]CGP-42112 exclusively labels AT_2, and not AT_1, receptors [21]. Binding to AT_2 receptors was the difference between [^{125}I]CGP-42112 binding and the binding remaining in adjacent sections incubated in the presence of 5 μM Ang II. Each animal was quantified independently.

After the incubation, the sections were washed four times for 1 min each in ice-cold 50 mM Tris–HCl buffer (pH 7.4), followed by a 30-s wash in ice-cold water, and dried under a stream of cold air. Sections were exposed to BioMax MR films (Eastman Kodak, Rochester, NY) together with [^{14}C]microscales (American Radiolabeled Chemicals, St. Louis, MO). Films were developed in ice-cold GBX developer (Eastman Kodak) for 4 min, fixed in Kodak GBX fixer for 4 min at 22 °C, and rinsed in water for 15 min. Optical densities of autoradiograms generated by incubation with the [^{125}I] ligands were normalized after comparison with [^{14}C] standards as described [22] and quantified by computerized microdensitometry using the Image 1.61 program (NIMH, Bethesda, MD). Films were exposed for different times depending on the amount of binding present, to obtain film images with optical densities clearly within the linear portion of the standard curve, and transformed to corresponding values of fmol/mg protein [23].

Kidney regions were identified in sections adjacent to those used for autoradiography, stained with hematoxylin–eosin (H&E) (Polysciences, Warrington, PA, USA).

2.3. In situ hybridization of Ang II receptor subtype mRNAs

To obtain rat AT_{1A}, AT_{1B}, and AT_2 receptor-specific riboprobes, partial fragments of full-length cDNA were subcloned into the polylinker site of the pBluescript KS^+ vector (Stratagene, La Jolla, CA). The rat (r) AT_{1A} cDNA was restricted with *Hae*III [24]. The restriction fragment of 368 bp [from nucleotides (nt) 1317–1684] was prepared and ligated into the *Eco*RV site of the vector. The fragment corresponded to the 3′UTR of the gene with a 35-bp ORF region that did not show any identity with rAT_{1B} cDNA ORF. The rAT_{1B} cDNA was restricted with *Hin*dIII and *Eco*RI [24]. The restriction fragment of 398 bp (from nt 1832–2229) was prepared and ligated into the *Hin*dIII–*Eco*RI site of the vector. The fragment corresponded to the 3′UTR of the gene. The rAT_2 cDNA was restricted with *Xba*I and *Bgl*II [24] and the fragment of 375 bp (from nt 1478–1852) was prepared and ligated into the *Xba*I–*Bgl*II site of the vector. The fragment corresponded to the 3′UTR of the gene. The subclones, rAT_{1A}-S2, rAT_{1B}-S1, and rAT_2, were confirmed by DNA sequencing.

For in vitro transcription of the ^{35}S-labeled antisense and sense (as a control) riboprobes, the subclones were linearized with *Hin*dIII or *Eco*RI for the rAT_{1A}-S2 and rAT_{1B}-S1 or with *Xba*I or *Eco*RI for the rAT_2, and then they were treated with T3 or T7 RNA polymerase, respectively. In vitro transcription was performed using the RNA labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described [24]. The labeling of riboprobes was monitored with liquid scintillation counting and controlled by a preliminary experiment using adrenal gland as a positive control.

To perform in situ hybridization, sections were fixed in 4% paraformaldehyde for 10 min, acetylated for 10 min in 0.1 M triethanolamine HCl, pH 8.0, containing 0.25% acetic anhydride, dehydrated in alcohols, and air-dried. Each section was covered with 150 μl hybridization buffer containing 50% formamide, 0.3 M NaCl, 2 mM EDTA, 20 mM Tris (pH 8.0), 1× Denhardt's solution, 10% dextran sulfate, 100 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, 150 mM dithiothreitol, 0.1% SDS, and 40,000 cpm/μl sense or antisense probe. Sections were hybridized overnight at 54 °C, treated with 40 μg/ml ribonuclease A (Sigma) for 30 min, and washed in sodium chloride/sodium citrate with increasing stringency. After a final wash in 0.1× standard saline citrate (SSC) at 65 °C for 60 min, sections were dehydrated through alcohols and exposed to Hyperfilm-3H (Amersham Pharmacia Biotech) along with ^{14}C-labeled microscales (American Radiolabeled Chemicals) for 7 days. Films were developed as described above. The intensities of hybridization signals were quantified as nanocuries per gram tissue equivalent by measuring optical film densities using the NIH Image 1.61 program after calibration with the ^{14}C-labeled microscales [22]. Nonspecific hybridization was analyzed using sense (control) probes.

2.4. Immunohistochemistry

Kidney sections (6 μm) were cut with a cryostat, air-dried, fixed to polysine slides (Erie Scientific) for 10 min in cold acetone, rinsed in PBS and incubated 10 min in 0.03% H_2O_2 at room temperature. Sections were rinsed again in PBS, incubated for 60 min in 10% goat serum in PBS and incubated with anti-rat eNOS mouse monoclonal antibody (Transduction Laboratories, Lexington, KY, USA; dilution 1:100) for 60 min at room temperature. The expression of the eNOS in renal cortical arteries was determined by optic microscopic observation. The antibody was visualized with the Dako Envision System (Dako, Carpinteria, CA), by using the diaminobenzidine chromogen in a peroxidase reaction,

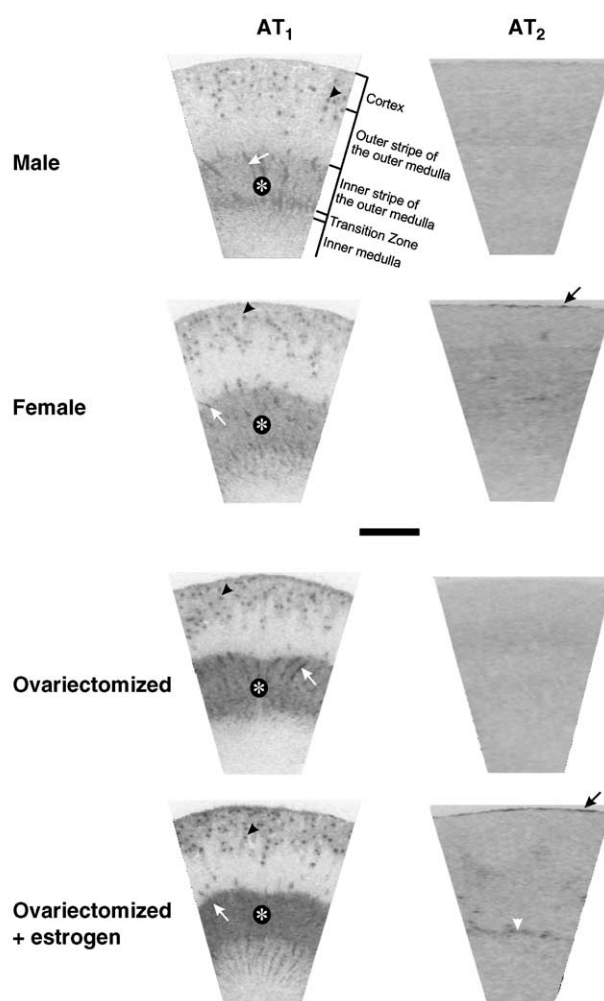

Fig. 1. Autoradiography of renal Angiotensin II AT_1 and AT_2 receptor binding in Wistar Hanover rats. Figures on the left correspond to AT_1 receptor binding. Black arrowheads indicate intense labeling of cortical glomeruli. There was no binding in the outer layer of the outer medulla. White asterisks indicate intense labeling in the inner stripe of the outer medulla. Highest binding was present in the vasa recta bundles, indicated by white arrows. Figures on the right correspond to AT_2 receptor binding. See Materials and methods. Black arrows indicate AT_2 receptor binding in the capsule. The white arrowhead indicates high AT_2 receptor binding in the transition zone. Bar is 1.5 mm.

and specimens were counterstained with hematoxylin. A total of 10 different arterial sections were studied from five animals per group, by an observer unaware of the group and treatment of the animals.

2.5. Determination of cGMP and prostaglandin E_2 content

For determination of cGMP and prostaglandin E_2 (PGE_2) content, we dissected the inner medulla including the transition zone and homogenized the tissue in phosphate saline buffer pH 7.4 containing 200 μM indomethacin and 500 μM IBMX. We determined the PGE_2 content by RIA using a commercially available kit (Biotrack, Amersham Pharmacia Biotech) and cGMP using a RIA kit (Biotrack, Amersham Pharmacia Biotech) after extraction with aqueous ethanol, according to the specifications of the manufacturers. Amounts of tissue homogenates were adjusted in preliminary experiments to be on the linear range for each RIA curve.

2.6. Statistics

Data are presented as means ± S.E.M. We used one-way ANOVA, followed by post-hoc analysis using the

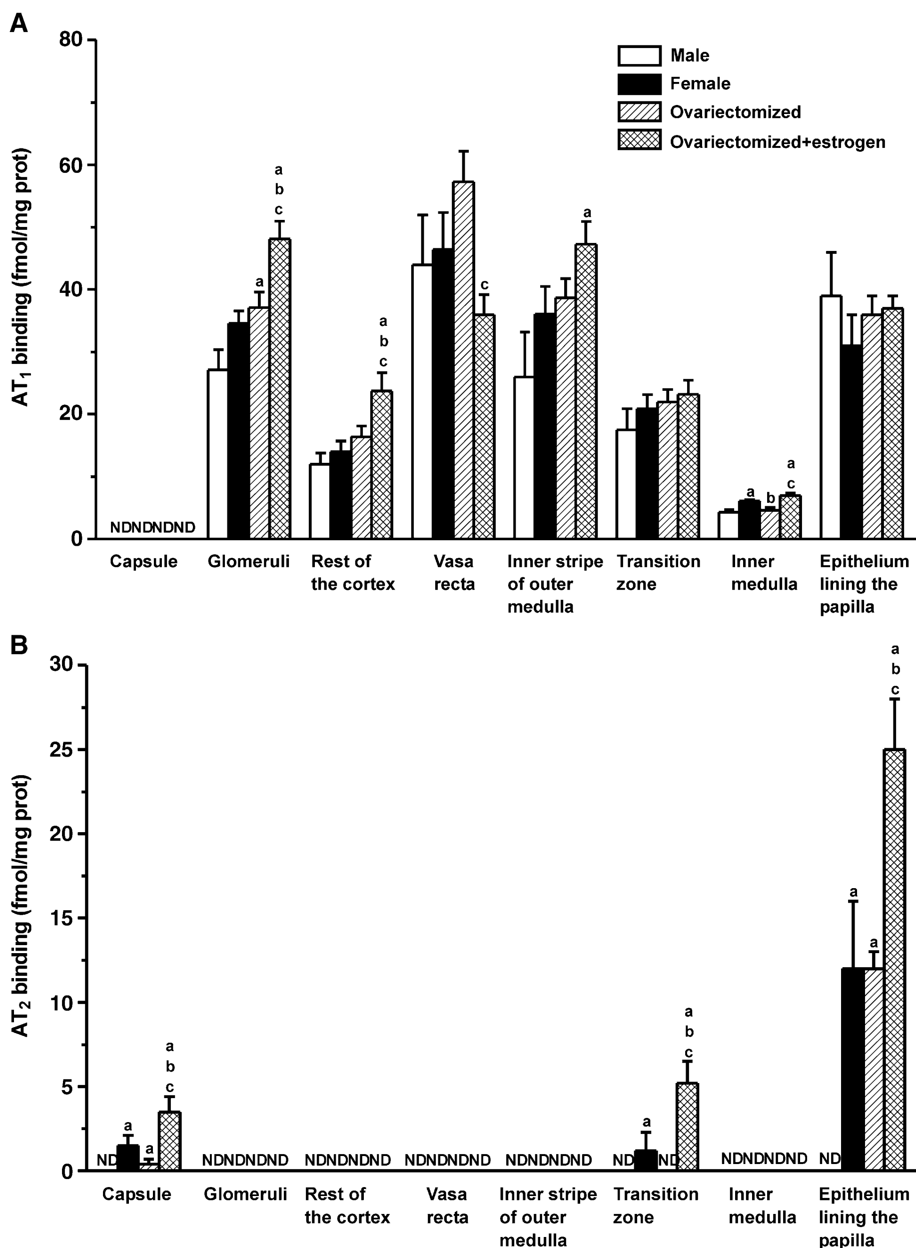

Fig. 2. Quantitative autoradiography of AT_1 and AT_2 Angiotensin II receptor binding in the kidney. (A) Specific binding to AT_1 receptors. (B) Specific binding to AT_2 receptors. See Materials and methods. Values are mean ± S.E.M. for groups of six animals measured individually. [a]Different from males, [b]different from females, [c]different from ovariectomized ($p < 0.05$).

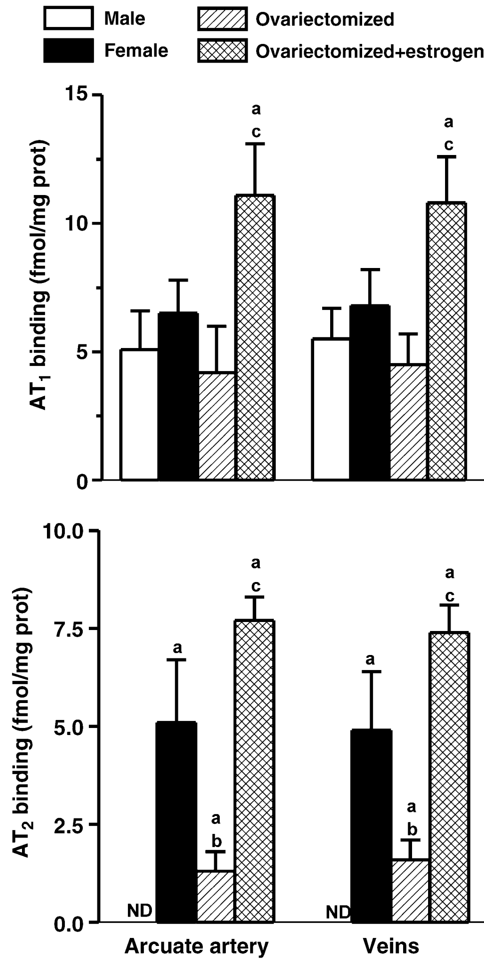

Fig. 3. Quantitative autoradiography of AT_1 and AT_2 Angiotensin II receptor binding in renal vessels. C. Specific binding to AT_1 receptors. D. Specific binding to AT_2 receptors. See Materials and methods. Values are mean ± S.E.M. for groups of six animals measured individually. [a]Different from males, [b]different from females, [c]different from ovariectomized ($p < 0.05$).

Newman–Keuls multiple comparison test to assess the significance of differences among groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Distribution and expression of renal Angiotensin II receptor types in male and female rats

3.1.1. AT_1 receptors

AT_1 receptors were the predominant Ang II receptor type in the kidney of both male and female rats, and the receptor binding distribution was widespread (Figs. 1 and 2). The receptor concentration was dependent on the area examined. In Wistar–Hanover rats, high numbers of AT_1 receptors were identified in the glomeruli, vasa recta bundles, inner stripe of the outer medulla, and the epithelium lining the papilla; intermediate receptor numbers were expressed in the rest of the cortex and transition zone, and low numbers in the inner medulla and arcuate arteries and veins; there were no AT_1 receptors in the kidney capsule (Figs. 1–3). In Wistar–Kyoto rats, the number of AT_1 receptors was about one-half of that of Wistar–Hanover rats, and there were no AT_1 receptors in the capsule or the inner medulla (results not shown).

Although the number of AT_1 receptors was generally higher in females than in males, the differences were not statistically significant, with the exception of the inner medulla of the Wistar–Hanover rats (Fig. 2) and the inner stripe of the outer medulla of Wistar–Kyoto rats, where the number of AT_1 receptors in female rats was significantly higher than that observed in males (males 5.3 ± 0.4 fmol/mg prot, females, 10.0 ± 1.6 fmol/mg prot, $p < 0.05$).

The expression of AT_{1A} receptor mRNA predominated in the rat kidney (Fig. 4, Table 1) and it was consistent with the distribution of AT_1 receptor binding (Figs. 1 and 4, Table 1). AT_{1A} mRNA level was highest in the vasa recta bundles and in the inner stripe of outer medulla. In the cortex, highest localized hybridization was in kidney glomeruli, and diffuse

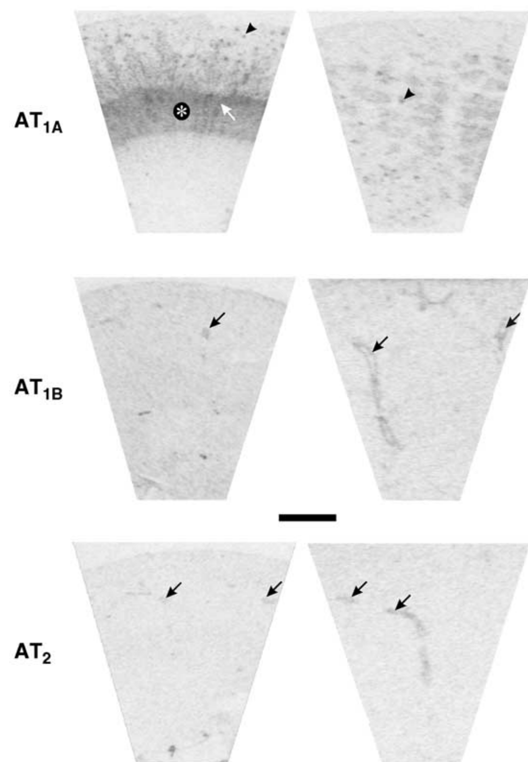

Fig. 4. In situ hybridization of AT_{1A}, AT_{1B} and AT_2 receptor mRNA in the kidney of Wistar Hanover rats. Sections correspond to an ovariectomized rat treated with estrogen. Figures on the left: Sections taken from the renal midsection. Figures on the right: sections taken lateral to the renal midsection. AT_{1A}: hybridization with a 3′UTR AT_{1A} receptor antisense ^{35}S riboprobe. AT_{1B}: hybridization with a 3′UTR AT_{1B} receptor antisense ^{35}S riboprobe. AT_2: hybridization with a 3′UTR AT_2 receptor antisense ^{35}S-labeled riboprobe. Arrowheads indicate AT_{1A} mRNA in the glomeruli. Black arrows indicate AT_{1B} and AT_2 mRNA hybridization in arcuate arteries and veins. The white arrow indicates AT_{1A} hybridization in the vasa recta bundles. The white asterisk denote AT_{1A} mRNA hybridization in the inner stripe of the outer medulla. Bar is 1.5 mm.

Table 1
Renal AT_{1A} mRNA in Wistar–Hanover rats

	AT_{1A} mRNA (nCi/g)			
	Glomeruli	Rest of cortex	Vasa recta bundles	Inner stripe outer medulla
Male	382 ± 62	85 ± 19	724 ± 95	412 ± 84
Female	297 ± 17	89 ± 13	709 ± 125	503 ± 83
Ovariectomized	244 ± 24	61 ± 11	709 ± 144	395 ± 73
Ovariectomized + estrogen	330 ± 27	56 ± 6.6	545 ± 49	343 ± 27

hybridization was present in the rest of the cortex (Fig. 4, Table 1). The expression of AT_{1A} mRNA was lower in Wistar–Kyoto when compared to Wistar–Hanover in all kidney areas (results not shown). There was no difference in the AT_{1A} mRNA expression between male and female rats (Table 1). No AT_{1A} mRNA expression could be detected in kidney vessels. Conversely, the expression of AT_{1B} mRNA was limited to kidney vessels, in particular the arcuate arteries and veins (Fig. 4, Table 2). No differences in AT_{1B} mRNA expression were noted between male and female Wistar–Hanover rats (Table 2) and the AT_{1B} mRNA expression was similar in Wistar–Kyoto rats (results not shown).

3.1.2. AT_2 receptors

The number of the AT_2 receptors was much lower than that of AT_1 receptors. There was no AT_2 receptor expression in the kidney of male rats, with the exception of low AT_2 expression in the epithelium lining the papilla of Wistar–Kyoto male rats (results not shown). Expression of AT_2 receptor binding was restricted to the kidney capsule, the transition zone, the epithelium lining the papilla, and the arcuate arteries and veins of female rats (Figs. 1, 2 and 5). The expression of AT_2 receptor binding was similar in Wistar–Kyoto female rats (results not shown).

We detected AT_2 receptor mRNA only in arcuate arteries and veins from female Wistar–Hanover rats (Fig. 3, Table 2), and similar values were found in Wistar–Kyoto female rats (results not shown). There was no AT_2 receptor mRNA in vascular structures from male animals.

Table 2
Renal AT_{1B} and AT_2 mRNA in arcuate vessels of Wistar Hanover rats

	mRNA (nCi/g)			
	AT_{1B}		AT_2	
	Arcuate artery	Arcuate veins	Arcuate artery	Arcuate veins
Male	46 ± 7.5	47 ± 7.3	ND	ND
Female	61 ± 10	63 ± 9.6	39 ± 5.9[a]	40 ± 6.0[a]
Ovariectomized	52 ± 6.5	50 ± 6.2	34 ± 4.5[a]	32 ± 4.1[a]
Ovariectomized + estrogen	68 ± 4.5	65 ± 5.0	46 ± 6.5[a]	45 ± 7.0[a]

ND: non-detectable.
[a] Different from male.

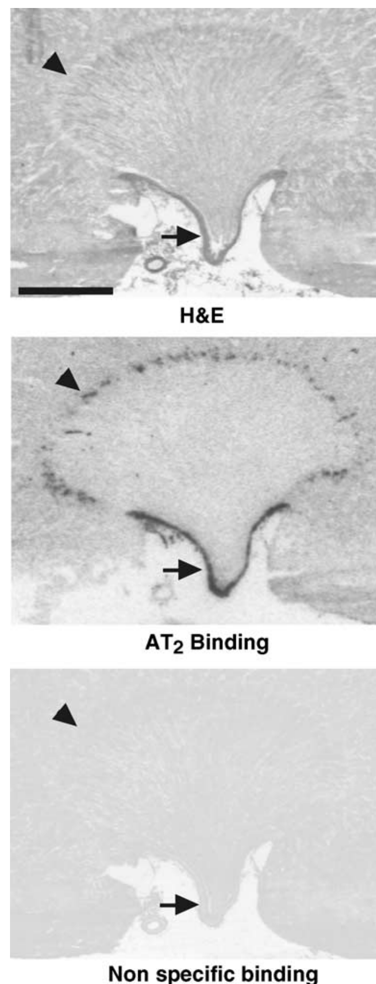

Fig. 5. Autoradiography of AT_2 receptors in the renal papilla. Sections are from an ovariectomized Wistar–Hanover rat treated with estrogen. Upper figure: on the left: hematoxylin–eosin staining (H&E). Middle figure: specific AT_2 receptor binding. Lower figure: Non-specific binding. See Materials and methods. Black arrows indicate AT_2 receptor binding in the epithelium lining the papilla. The black arrowheads indicate high AT_2 receptor binding in the transition zone between the inner stripe of the outer medulla and the inner medulla. Bar is 2 mm.

3.2. Expression of Angiotensin II receptor types after ovariectomy and estrogen replacement

3.2.1. AT_1 receptors

In Wistar–Hanover rats, ovariectomy did not significantly change the expression of AT_1 receptor binding, with the exception of a decrease in receptor binding in the inner medulla (Fig. 2). In Wistar–Kyoto rats, however, ovariectomy increased AT_1 receptor binding when compared to the receptor concentrations found in intact females (glomeruli: 15.9 ± 1.1 and 23.1 ± 3.2; vasa recta bundles: 27.8 ± 2.4 and 42.2 ± 4.0; transition zone: 7.5 ± 1.2 and 13.3 ± 1.9 fmol/mg protein, for female and ovariectomized rats, respectively, $p < 0.05$).

Estrogen replacement elevated the hormone to supraphysiological levels (females, 8.8 ± 4.2, ovariectomized + estro-

gen 187.8 ± 25.5 pg/ml) about two times higher than levels found at the ovulatory peak [25] to levels similar to those found during pregnancy [26]. Estrogen treatment of ovariectomized rats significantly increased the number of AT_1 receptors when compared to ovariectomized in glomeruli, rest of cortex, inner medulla, and arcuate arteries and veins of Wistar–Hanover rats (Fig. 2), and in glomeruli, vasa recta bundles, inner stripe of the outer medulla, the transition zone, and arcuate arteries and veins in Wistar–Kyoto rats (results not shown). The exception was the vasa recta in Wistar–Hanover rats, were estrogen treatment significantly decreased the AT_1 receptor expression when compared to ovariectomized rats (Fig. 2). While estrogen treatment modified the expression of AT_1 receptor binding, there were no statistical differences in the expression of AT_{1A} mRNA in male, female, OVX and OVX-estrogen-treated rats in Wistar–Hanover rats (Table 1) or Wistar–Kyoto rats (results not shown).

3.2.2. AT_2 receptors

The expression of AT_2 receptors was also estrogen-dependent. In Wistar–Hanover rats, AT_2 receptor expression was significantly decreased by ovariectomy in the transition zone and the arcuate arteries and veins (Figs. 1–3). In Wistar–Kyoto rats, ovariectomy decreased AT_2 receptor binding only in the epithelium lining the papilla (results not shown).

Estrogen treatment restored, and even increased, AT_2 receptor binding in the capsule, transition zone, the epithelium lining the papilla, and the arcuate vessels Wistar–Hanover rats (Fig. 1, 2 and 5) and Wistar–Kyoto rats (results not shown). AT_2 mRNA was detected by in situ hybridization arcuate arteries and veins from female, ovariectomized and ovariectomized-estrogen replaced rats, and the differences in AT_2 mRNA expression were not significant between these groups (Fig. 4 and Table 2).

3.2.3. eNOS expression in renal cortical arteries

We assessed the expression of eNOS expression in renal cortical arteries from Wistar–Hanover rats. A total of three sections per animal were studied. The intensity of the immunostaining was analyzed by investigators unaware of the treatment received by the individual animals, and estimated qualitatively as low, medium or high intensity. There was a marked increase in eNOS immunostaining in ovariectomized rats after estrogen replacement (Fig. 6).

3.3. PGE_2 and cGMP content in the renal medulla

We studied the effects of estrogen replacement on PGE_2 and cGMP content in the inner medulla including the transition zone of ovariectomized Wistar–Hanover rats. In

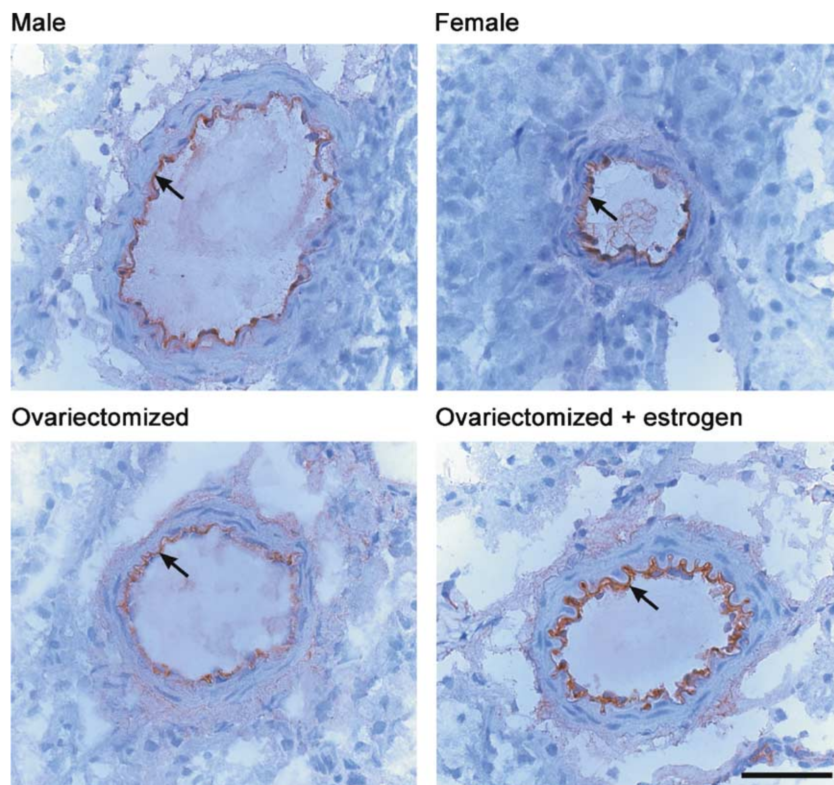

Fig. 6. Immunohistochemical localization of eNOS in renal cortical arteries of Wistar–Hanover rats. eNOS was localized to the endothelium of renal cortical arteries. Note the increased expression of eNOS in female rats when compared to male rats, the decreased expression in ovariectomized (OVX) rats, and the increased expression when OVX rats were treated with estrogen (OVX + E). Bar is 50 μm.

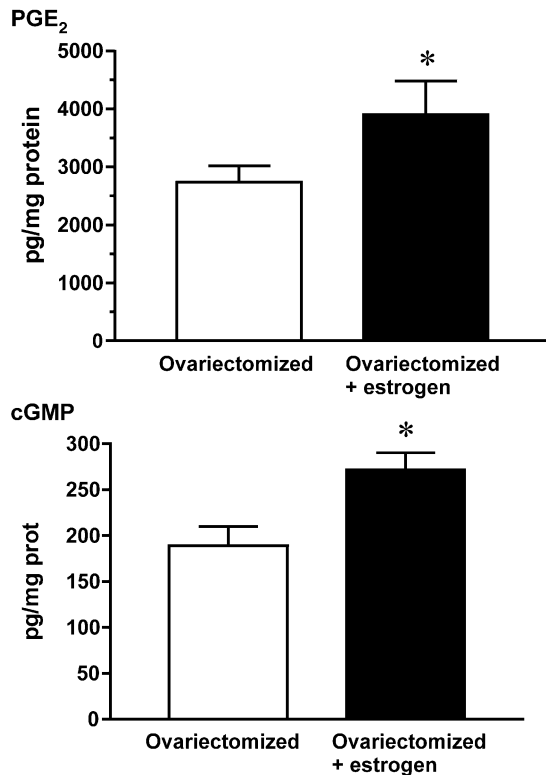

Fig. 7. PGE_2 and cGMP content in the inner medulla of Wistar–Hanover rats. Results are means ± S.E.M. of groups of six rats, measured individually. OVX:ovariectomized. OVX + E: ovariectomized treated with estrogen. *$p < 0.05$ vs. OVX rats.

these animals, estrogen replacement significantly increased the concentrations of PGE_2 and cGMP when compared to ovariectomized rats (Fig. 7).

4. Discussion

Our findings confirm the predominance of AT_1 receptors in the kidney of the male rat, with relatively minor differences across strains, and their selective localization to glomeruli, other cortical structures, vasa recta bundles, inner stripe of the outer medulla, transition zone, inner medulla, epithelium lining the papilla, and arcuate arteries and veins [5,27], correlating with the sites where the peptide exerts its major physiological effects.

In rodents, there are two AT_1 receptor subtypes, AT_{1A} and AT_{1B}, encoded by two distinct genes, differentially expressed and regulated [28–31]. Using in situ hybridization, we confirmed that in the kidney of the male Wistar–Hanover and Wistar–Kyoto rats, the main AT_1 receptor subtype was the AT_{1A} receptor [3,32]. Expression of AT_{1B} receptor mRNA was only found in the kidney vasculature, suggesting that while AT_{1A} receptors were involved in regulation of sodium and water reabsorbtion and filtration rates, AT_{1B} receptors may play a significant role in the control of renal blood flow.

Renal AT_1 receptor expression was estrogen-dependent in several kidney structures. Because the need to visualize selective binding to Ang II receptor types precluded the use of saturating ligand concentrations [20], our results could indicate changes in ligand affinity or in receptor number. Ovariectomy affected AT_1 receptor expression in selected kidney areas, and these effects were strain dependent. For the most part, ovariectomy did not modify the AT_1 receptor expression in Wistar–Hanover rats, but increased AT_1 receptor expression in the glomeruli, vasa recta bundles and transition zone of Wistar–Kyoto rats. For this reason, we believe that other hormonal factors could exert influence upon the AT_1 receptor expression in selected kidney areas, and that the estrogen dependency of AT_1 receptor expression was to a certain extent strain-dependent.

There was a significant increase in AT_1 receptor expression when ovariectomized rats received estrogen replacement. We administered estrogen at a dose that increases its levels about twofold over those found during the ovulatory peak [25] and similar to those found during pregnancy [26], and we did not study the influence of cyclic variations in estradiol production on the expression of Ang II receptor types. However, the use of ovariectomy as well as estradiol treatment models addresses both endogenous and exogenous estrogen effects. In female rats, neutering reduced the rate of weight gain, and estrogen replacement prevented this effect [33]. However, these changes were not major two weeks after surgery [33]. For this reason, it is not likely that our results are dependent on alterations in body weight.

Estrogen can directly modulate AT_1 receptor transcription through the estrogen response elements (ERE) in the 5′-flanking region of the receptor genes [34]. However, estrogen increased AT_1 receptor binding but did not change AT_{1A} or AT_{1B} receptor mRNA expression. In adrenal gland, estrogen decreased AT_1 receptor number by reducing AT_{1A} and AT_{1B} receptor translation by regulation of cytosolic proteins that bind to the 5′leader sequence of the receptor mRNA [35,36] and a similar regulatory mechanism could occur in the kidney. It is also possible that estrogen affects receptor turnover or receptor affinity, rather than receptor transcription or mRNA stability.

There were differences in AT_2 receptor expression between rats, as reported here, and mice of the CB57BL/6J strain [17], with rats expressing lower AT_2 receptor numbers than mice. We found no AT_2 receptor binding in the kidney of male rats, with the exception of very low amounts of binding in the epithelium lining the papilla in Wistar–Kyoto rats. This is in agreement with previous data in the rat [10,11]. Conversely, in male mice AT_2 receptors were present in glomeruli and tubular structures [17]. We found similar differences in renal expression of AT_2 receptors between mice and rats of other strains (Sprague–Dawley and Spontaneously Hypertensive Rats, 129 Ola, 129 Ola/C57BL/6J, 129Sv/J/C57BL/6J mice, results not shown), indicating that differences between

mice and rats may occur in most, if not all strains of both species.

The expression of AT_2 receptors in female rats was more restricted than that in female mice, and it was also estrogen-dependent, as revealed by increased receptor expression in the renal capsule, transition zone, epithelium lining the papilla, and arcuate vessels of ovariectomized rats after hormone replacement. The estrogen-dependent increase in the expression of AT_2 receptors in the transition zone is of special interest, since the thick ascending limb of the loop of Henle is the predominant anatomical structure in this area, involved in the regulation of NaCl transport and divalent cations [37], is influenced by vasopressin [38] and estrogen treatment impairs the renal response to this neurohormone [39]. Our results suggest that AT_2 receptors are involved in the regulation of ion transport and the tonicity of the interstitium.

We confirmed that the influence of estrogens on kidney AT_2 receptor expression is a general phenomenon for rodent species. Estrogens did not affect the expression of AT_2 receptor mRNA, and the promoter region of the AT_2 receptor does not contain the consensus ERE [40], suggesting that the hormone might regulate receptor translation, turnover or affinity, rather than transcription. However, we have shown that estrogen treatment increases AT_2 receptor mRNA in mice [17].

What is the consequence of the modulation of AT_1 and AT_2 receptors by estrogen? Estrogen produces complex changes in renal RAS, including increases in the concentration of Ang I and Ang I fragments without changes in renal Ang II and decreased renin secretion [41]. Our findings of increased glomerular AT_1 receptor binding, indicative of possible increased receptor function, can explain the negative influence in renin secretion, even in the presence of normal renal Ang II concentrations. However, the effects of estrogen on kidney RAS may differ according to the structure studied, because estrogen increases AT_1 receptors in renal cortex, medulla and vessels, but decreases AT_1 receptor number in the vasa recta.

Increased AT_1 receptor function induced by estrogen could reduce the glomerular filtration coefficient [2] contribute to the known sodium-retaining effect induced by estrogen [44,45] and could enhance the vasoconstrictor effect of Ang II in the medullary circulation [44,46].

Although Ang II, through AT_1 receptor stimulation is a potent vasoconstrictor, in conscious rats the net effect of changes in circulating Ang II within the physiological range on medullary blood flow is minimal [47], presumably due to the counteracting effects of different vasodilator factors. AT_2 receptor stimulation was proposed to balance AT_1 receptor stimulation, reducing the effect of AT_1-mediated responses, a mechanism of cross-talk [42,43]. The cross-talk hypothesis was based on studies in cultured cells, and most of the renal AT_1 and AT_2 receptors have been localized to different cell types [17]. Thus, it is likely that the interaction between renal receptor types is indirect rather than intracellular. In male rats, the absence of AT_2 receptors results in an absolute predominance of AT_1 receptor stimulation. In Wistar–Hanover female rats, ovariectomy increases and estrogen replacement restores the AT_1/AT_2 receptor ratio in arcuate arteries and veins. A more favorable balance in the direction of AT_2 receptor stimulation could increase flow-induced dilation in resistance arteries, improving regional renal blood flow and reducing blood pressure [48].

The mechanisms of vasodilation resulting from AT_2 receptor stimulation include an increased bradykinin production leading to NO release [49] and induction of PGE_2 formation [50]. We studied the inner medulla including the transition zone because of the relatively high AT_2 receptor concentration in this area. The increase in PGE_2 concentration in the renal medulla in ovariectomized rats after estrogen replacement agrees with previous reports [51] and the resulting vasodilation [42,52] can attenuate the vasoconstrictor effect of AT_1 receptor stimulation [42].

Additional mechanisms leading to vasodilation include, as reported here in estrogen-replaced ovariectomized rats, the increased in eNOS expression in renal cortical arteries, which is in agreement with previous reports of estrogen-induced increase in renal medullary eNOS [53] and the increase in cGMP levels, which can also be attributed to AT_2 receptor stimulation [54], and is mediated in the kidney by NO production and in turn dependent on bradykinin formation [49].

AT_2 receptor stimulation could counteract additional AT_1-receptor-mediated kidney functions. While Ang II retains sodium and blocks NO production by stimulation of AT_1 receptors [55], AT_2 receptor stimulation produces natriuresis, a process involving the participation of cGMP, an effect mediated in the kidney by NO production and in turn dependent on the formation of bradykinin [49]. Additional AT_1 receptor-mediated effects antagonized by AT_2 receptor stimulation include stimulation of cell growth [15], development of kidney fibrosis [56,57] and increased renal hypersensitivity to Ang II [58]. These effects of AT_2 could be mediated by PGE_2, a compound that inhibits growth of mesangial cells [59] and reduces the expression and secretion of collagen [60].

In conclusion, in rats, as was earlier reported in mice, the predominant Ang II receptor is the AT_1 receptor type. In rats, the predominant subtype is the AT_{1A} receptor, while AT_{1B} receptors are expressed in vascular structures. The AT_2 receptor expression is lower in rats than in mice, and it is not present in male rats with the exception of very low levels in the epithelium lining the papilla of Wistar–Kyoto rats, while it is clearly expressed in male mice. AT_1 and AT_2 receptor expression is partially estrogen-dependent, with region, species and strain differences. AT_{1A}, AT_{1B} and AT_2 Ang II receptors may play important and different roles in the kidney, depending on the species and strain studied, and their actions can be modulated by reproductive hormones. AT_{1A} and AT_2 receptors are probably involved in

sodium and water reabsorbtion and glomerular filtration in the female. In male rats, absence of AT_2 receptors suggests that this receptor is not necessary for the regulation of sodium and water reabsorbtion. AT_{1B} and AT_2 receptors may play significant roles in the regulation of renal blood flow. In renal arteries, estrogen can decrease the AT_1/AT_2 receptor ratio, favoring vasodilation, a shift that can contribute to explain the gender differences on the renal response to Ang II [61], the higher papillary blood flow and the slower progression of renal disease seen in females [61,62].

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