Estrogen upregulates renal angiotensin II AT\textsubscript{2} receptors

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Received 16 April 2002; accepted in final form 14 June 2002

Estrogen upregulates renal angiotensin II AT\textsubscript{2} receptors. Am J Physiol Renal Physiol 283: F934–F943, 2002; 10.1152/ajprenal.00145.2002.—AT\textsubscript{2} receptors may act in opposition to and in balance with AT\textsubscript{1} receptors, their stimulation having beneficial effects. We found renal AT\textsubscript{2} receptor expression in female mice higher than in male mice. We asked the question of whether such expression might be estrogen dependent. In male, female, ovariectomized, and estrogen-treated ovariotomized mice, we studied renal AT\textsubscript{1} and AT\textsubscript{2} receptors by immunocytochemistry and autoradiography, AT\textsubscript{2} receptor mRNA by RT-PCR, and cAMP, cGMP, and PGE\textsubscript{2} by RIA. AT\textsubscript{1} receptors predominated. AT\textsubscript{2} receptors were present in glomeruli, medullary rays, and inner medulla, and in female kidney capsule. AT\textsubscript{1} and AT\textsubscript{2} receptors colocalized in glomeruli. Female mice expressed fewer glomerular AT\textsubscript{1} receptors. Ovariectomy decreased AT\textsubscript{1} receptors in medullary rays and capsular AT\textsubscript{2} receptors. Estrogen administration normalized AT\textsubscript{1} receptors in medullary rays and increased AT\textsubscript{2} receptors predominantly in capsule and inner medulla, and also in glomeruli, medullary rays, and inner stripe of outer medulla. In medullas of estrogen-treated ovariotomized mice there was higher AT\textsubscript{2} receptor mRNA, decreased cGMP, and increased PGE\textsubscript{2} content. We propose that the protective effects of estrogen may be partially mediated through enhancement of AT\textsubscript{2} receptor stimulation.

ANG II REGULATES renal function in mammals by stimulation of specific, discretely localized ANG II AT\textsubscript{1} receptors (34), localized in the adult mammalian kidney, predominantly in glomeruli, with lower levels in renal cortical tubules, vasculature, medullary interstitial cells, and collecting ducts (13, 47). Renal AT\textsubscript{1} receptor stimulation produces sodium retention, vasoconstriction, decreased glomerular filtration rate, increased mesangial cell hypertrophy (1), and renal injury (27).

The function of the second ANG II receptor type, the AT\textsubscript{2} receptor, is controversial (11). While their expression in fetal kidney suggests a role during development (8), in the adult kidney of the male rat AT\textsubscript{2} receptors were reported to be absent when studied by autoradiography (8, 10) or detected only at low levels when studied by immunocytochemistry (44). In adult male mice, however, AT\textsubscript{2} receptors are clearly expressed and associated with blood vessels (47) and, in humans, AT\textsubscript{2} receptor mRNA is localized in blood vessels, tubular structures, and glomeruli (33), suggesting that AT\textsubscript{2} receptor expression is higher in mice and humans than in rats. These observations indicated a participation of AT\textsubscript{2} receptors in renal vascular flow regulation and perhaps other kidney functions (2, 7, 48). Recent evidence appears to indicate that renal AT\textsubscript{2} receptor stimulation dilates efferent arterioles (2), decreases mesangial cell hypertrophy (14), and is natriuretic (7), suggesting that AT\textsubscript{2} receptors may act in opposition to and in balance with AT\textsubscript{1} receptors and that their stimulation could have beneficial effects.

In preliminary experiments, we found that female mice expressed renal AT\textsubscript{2} receptors in numbers substantially higher than those present in male mice. We asked the question of whether such a differential expression might be estrogen dependent and could in any way be related to the postulated effects of estrogen replacement therapy, the prevention of development of hypertension, and the delayed progression of renal disease (16, 38).

The mode of action of estrogen on the cardiovascular system includes important and complex regulatory influences on the renin-angiotensin system (RAS) (49). Although estrogen stimulates the synthesis of the renin substrate angiotensinogen (9) and increases renal ANG II (6), it also suppresses plasma renin activity (49), decreases renal angiotensin-converting enzyme (ACE) (5), and downregulates AT\textsubscript{1} receptors (34) in vascular smooth muscle cells (43), pituitary gland (50), and adrenal cortex (45). However, no studies on gender differences in renal AT\textsubscript{2} receptors have been conducted.

To further clarify the role of estrogens in the kidney and their influence on the renal ANG II system, we studied the expression of renal ANG II receptor types in male, female, ovariotomized, and estrogen-treated ovariotomized mice.

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MATERIALS AND METHODS

Animals. Groups of six to eight 6-wk-old male, female, and ovariectomized female mice of the CB57BL/6J strain were obtained (Taconic, Germantown, NY) and kept under controlled conditions with free access to water and food, according to protocols approved by the National Institute of Mental Health Animal Care and Use Committee.

Experimental protocol. In a preliminary experiment, we compared normal nonoperated male and female mice. In experiment 1, we compared normal male and sham-operated female mice to confirm gender differences in expression of ANG II receptor types. In experiment 2, we compared sham-operated females implanted with a cholesterol pellet, ovariectomized mice (OVX) implanted with a cholesterol pellet, and OVX mice treated with estrogen (OVX+E) to study the influence of estrogen on ANG II receptor type expression. In experiment 3, we compared normal male, sham-operated females implanted with a cholesterol pellet, OVX mice implanted with a cholesterol pellet, and OVX+E mice to study cyclic nucleotide and PGE2 levels in the inner medulla. Female mice were not separated into stages of the reproductive cycle. Fourteen days after OVX or sham operation, female mice were implanted subcutaneously with 17β-estradiol or cholesterol placebo pellets (1.7 mg/pellet, 60 days release, ∼28 µg 17β-estradiol/mouse−1·day−1, estimated blood level >900 pg/ml; Innovative Research, Sarasota, FL) under pentobarbital sodium anesthesia (30 mg/kg ip). 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.

Quantitative autoradiography of ANG II receptor types. For binding studies, consecutive 0.5-µm-thick kidney sections were cut in a cryostat at −20°C, thaw-mounted on gelatin-coated slides, dried overnight in a desiccator at 4°C, and kept at −80°C until use. 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 2 h at 22°C in fresh buffer, prepared as above with the addition of 50 µM Plummer’s inhibitor (Calbiochem, La Jolla, CA), 100 µM phenylmethylsulfonyl fluoride (Sigma), 500 µM phentolamine (Sigma), and 0.5 nM 125I-[Sar1]ANG II (Peninsula Laboratories, Belmont, CA), iodinated by the Pep- tide Radioiodination Service Center, Washington State University, Pullman, WA) to a specific activity of 2,176 Ci/mmol to determine total binding. After 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 14C 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 125I ligands were normalized after comparison with 14C standards as described (36) and quantified by computerized microdensitometry using the Image 1.61 program (National Institute of Mental Health, 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 femtometers per milligram protein (36, 40). Each animal was quantified independently.

Binding of 125I-[Sar1]ANG II to AT2 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 AT1 receptors. Binding to AT1 receptors was the difference between total binding and the binding remaining in adjacent sections incubated in the presence of an excess concentration of losartan. AT1 receptor binding was the binding selectively displaced by losartan in our experiments. Binding of 125I-[Sar1]ANG II to AT2 receptors was determined as the difference between total binding and binding in adjacent sections incubated in the presence of 10−6 M PD-123319 (Sigma) to selectively displace binding to AT2 receptors. AT2 receptor binding was the binding selectively displaced by PD-123319. The concentrations of the AT1 and AT2 receptor-selective ligands were chosen to give maximum-specific displacement (17, 46). Nonspecific binding was determined by incubating consecutive sections with 5 × 10−6 M labeled ANG II (Peninsula). We quantified binding for every structure analyzed by subtracting from consecutive sections the value obtained after selective displacement from total binding (measured without the displacer).

In addition, binding of 125I-CPG-42112 (specific activity 2,176 Ci/mmol, Peptide Radioiodination Service Center) was performed in another set of adjacent sections to confirm the presence of AT2 receptors. At the concentrations used, 125I-CPG-42112 exclusively labels AT2, and not AT1, receptors (18). 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 in fresh buffer containing 0.2 nM 125I-CPG-42112. To determine specific binding to AT2 receptors, consecutive sections were incubated in the presence of 10−6 M PD-123319 to selectively displace binding to AT2 sites. Binding to AT2 receptors was the binding displaced by PD-123319. Nonspecific binding was determined by incubating consecutive sections with 5 × 10−6 M ANG II (Peninsula). Kidney regions were identified in sections adjacent to those used for autoradiography, stained with hematoxylin-eosin (Polysciences, War- rington, PA).

Emulsion autoradiography. To further localize AT2 receptors, 125I-CPG-42112 binding was performed in 6-µm-thick kidney sections. After binding experiments, sections were fixed for 60 min in paraformaldehyde vapors at 80°C and dipped in photo emulsion (Eastman Kodak). After exposure for 1 day-2 wk, sections were developed in Kodak D-19 developer, counterstained with hematoxylin-eosin, and studied under darkfield microscopy.

Immunofluorescence. We performed dual immunofluorescence on 8-µm-thick frozen sections fixed in acetone (56). First, we used a monoclonal antibody against the third intracellular loop of the human AT1 receptor, amino acids 229–246 (4H2), at a 1:100 dilution for 1 h at room temperature (12). The specificity of the antibody was validated by the dot-blot assay, by Western blot analysis of whole adrenal protein, and by immunohistochemistry in sections of the rat adrenal gland. The AT1 receptor antibody reacted with both the AT1A and AT1B peptides (amino acids 229–246), and there was no cross-reactivity against the AT2A peptide (amino acids 314–330). In addition, the AT1 receptor antibody detected a prominent band with an apparent molecular mass of 73 kDa, consistent with that of the AT1 receptor, and detected the
presence of AT<sub>1</sub> receptors in the rat adrenal cortex and medulla (12). The AT<sub>1</sub> receptor antibody was labeled with FITC-conjugated goat anti-mouse IgG (H+L, Jackson ImmunoResearch, West Grove, PA), detected as green. Second, we used a goat polyclonal antibody against the COOH terminus of the AT<sub>2</sub> receptors (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution for 45 min at room temperature according to the manufacturer’s protocol. The AT<sub>2</sub> receptor antibody was labeled with tetramethyl rhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (H+L, Jackson ImmunoResearch), detected as red. Omitting the primary antibody and replacing it with nonimmune serum determined the specificity of immunoreactivity. Sections were mounted with Vectashield (Vector Lab, Burlingame, CA) and examined in a light fluorescent microscope using three different filters for FITC (green) for AT<sub>1</sub> receptors, rhodamine (red) for AT<sub>2</sub> receptors, and together for colocalization, recognized as yellow or orange.

AT<sub>2</sub> receptor mRNA. AT<sub>2</sub> receptor mRNA was determined in kidney medulla dissected freehand under a microscope by RT-PCR. Total RNA was prepared separately from five to six kidney medullas from each group (Qiagen). First-strand cDNA was synthesized from 2 μg of total RNA/sample with an oligo-dT primer and SuperScript II RT (GIBCO BRL). The resultant cDNAs were amplified by PCR using the following primers: AT<sub>2</sub> receptor primer 5′-CCA GCA GCC GTC CTT TTG ATA A-3′ (sense); 5′-GTA ATT CTG TTC CCA TAG C-3′ (antisense); GAPDH 5′-TCC ATG ACA ACT TTG GCA TC-3′ (sense); and 5′-CAT GTC AGA TCC ACC ACG GA-3′ (antisense). Amplification conditions were as follows: denaturation at 94°C for 1 min; annealing at 53°C (AT<sub>2</sub> receptor) or 55°C (GAPDH) for 1 min; and extension at 72°C for 1 min for 30 cycles, with a final extension step at 72°C for 7 min. Reaction conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction, as determined by preliminary experiments. The reaction was linear between 27 and 35 cycles when 2 μl of cDNA were used. The amplification products were separated on 2% agarose gels and stained with ethidium bromide. Band intensities were quantified by computer densitometry (National Institutes of Health Image, 1.6), using the expression of GAPDH as a control.

Determination of cAMP, cGMP, and PGE<sub>2</sub> content. For determination of cAMP, cGMP, and PGE<sub>2</sub> content, we dissected the inner medulla and homogenized the tissue in phosphate saline buffer, pH 7.4, containing 200 μM indomethacin and 500 μM IBMX. We determined the PGE<sub>2</sub> content by RIA using a commercially available kit (Biotrack, Amersham Pharmacia Biotech, Piscataway, NJ) and cAMP and cGMP using an RIA kit (Biotrack, Amersham Pharmacia Biotech) after extraction with aqueous ethanol, according to the manufacturer’s specifications. Amounts of tissue homogenates were adjusted in preliminary experiments to be on the linear range for each RIA curve.

Statistics. Data are means ± SE. We used Student’s t-test (Fig. 1) and one-way ANOVA followed by post hoc analysis using the Newman-Keuls multiple comparison test (Fig. 2; see also Figs. 6 and 7) to assess the significance of differences among groups. P < 0.05 was considered statistically significant.

RESULTS

Localization and expression of ANG II receptor types in male and female mouse kidney. In a preliminary experiment using normal, nonoperated male, and female mice, we found that female mice, but not male mice, expressed AT<sub>2</sub> receptors in the renal capsule (2.0 ± 0.6 fmol/mg protein) and that the number of AT<sub>2</sub> receptors in the inner medulla was significantly higher in female mice compared with male mice (2.1 ± 0.7 and 0.5 ± 0.1 fmol/mg protein for female and male mice, respectively, P < 0.05). Conversely, male mice expressed a higher level of AT<sub>1</sub> receptor binding in glomeruli compared with female mice (98 ± 5 and 85 ± 7 fmol/mg protein for male and female mice, respectively, P < 0.05).

To confirm and expand these observations, we performed a more complete study to compare male, sham-operated female, OVX-, and OVX+E-treated mice. The expression of ANG II receptor types in the male and sham-operated female kidney did not differ from that found in our preliminary study. AT<sub>1</sub> receptor expression predominates in both male and sham-operated female kidneys. The highest AT<sub>1</sub> receptor expression was noted in the glomeruli, followed by the rest of the cortex, medullary rays, inner stripe of the outer medulla, and inner medulla (Figs. 1 and 3). In glomeruli, sham-operated female mice expressed lower AT<sub>1</sub> receptor numbers than males (Fig. 1). There was no AT<sub>1</sub> receptor expression in the kidney capsule (Figs. 1 and 3).

Both male and sham-operated female mice expressed kidney AT<sub>2</sub> receptors, at concentrations much
lower than those of AT1 receptors (Figs. 1 and 3) in the glomeruli, medullary rays, and inner medulla. There were no AT2 receptors in the rest of the cortex or in the inner stripe of the outer medulla (Figs. 1 and 3). Concentrations of AT2 receptors were similar in the glomeruli and medullary rays of male and female mice (Figs. 1 and 3). In the inner medulla, female mice expressed higher numbers of AT2 receptors than did males (Figs. 1 and 3). Only female mice expressed AT2 receptors in the kidney capsule (Figs. 1 and 3).

Expression of ANG II receptor types after ovariectomy and estrogen replacement. OVX mice did not display major changes in AT1 receptor expression. The only significant change was a decrease in AT1 receptor number in the medullary rays, which was reversed in OVX+E mice (Fig. 2).

Conversely, ovariectomy and ovariectomy plus estrogen replacement resulted in some dramatic changes in renal AT2 receptor expression. In the renal capsule, ovariectomy eliminated the expression of AT2 receptors, while in OVX+E mice, their expression was enhanced 10-fold over the levels found in intact females (Figs. 2 and 3). In the glomeruli and medullary rays, small, but not statistically significant, decreases in AT2 receptor expression occurred after ovariectomy (Fig. 2). These changes were reversed in OVX+E mice to a level higher than that present in intact females (Fig. 2). Moreover, the inner stripe of the outer medulla, which did not express AT2 receptors in sham-operated female or OVX mice, expressed a significant receptor number in OVX+E mice (Figs. 2 and 3). The most impressive change in AT2 receptor expression, however, occurred in the inner medulla. In this region, although the number of AT2 receptors was not significantly changed by ovariectomy, the receptor expression was increased over 60-fold in OVX+E mice (Figs. 2 and 3).

Emulsion autoradiography of renal AT2 receptors. Emulsion autoradiography of binding of 125I-CGP-42112 to AT2 receptors revealed localization of binding in sham-operated female mice to the renal capsule, glomeruli, medullary rays, and inner medulla (Fig. 4).

Localization of ANG II receptor subtypes by immunocytochemistry. Dual immunocytochemistry with antibodies specific for AT1 and AT2 receptors revealed colocalization of AT1 and AT2 receptors in glomerular capillary loops, particularly in podocytes enveloping glomerular capillaries in sham-operated female mice (Fig. 5).

AT2 mRNA in the renal medulla. In the inner medulla, sham-operated female mice express higher AT2 receptor mRNA than males. Ovariectomy decreases AT2 receptor mRNA to levels no different from those in males. AT2 mRNA expression in OVX+E mice was significantly higher than that in male or OVX mice (Fig. 6).

cAMP and cGMP content in the renal medulla. The content of cAMP was not different in the renal medulla of male, sham-operated female, OVX, and OVX+E mice. Values were 1,000 ± 168, 957 ± 139, 1,075 ± 91, and ± 1,149 ± 247 pg/mg protein for males, females, OVX, and OVX+E mice, respectively. Conversely, in OVX+E mice, the cGMP content of the inner medulla significantly decreased (Fig. 7).

PGE2 content in the renal medulla. PGE2 content in the renal medulla of sham-operated female mice was higher than that in male and OVX mice, but the differences were not statistically significant (Fig. 7). On the other hand, PGE2 content in the kidney medulla of OVX+E mice was higher than that of any other group (Fig. 7).

DISCUSSION

We report that estrogen administration increases renal AT2 receptor number in the inner medulla by two orders of magnitude and significantly increases AT2 receptors in many other kidney structures. This is the first report of a profound effect of a reproductive hormone on the expression of renal AT2 receptors. We administered a supraphysiological dose of estradiol, resulting in blood levels in excess of those present in
normal mice, and we did not study the influence of cyclic variations in estradiol production in AT₂ receptor expression. However, the use of both ovariectomy as well as estradiol treatment models addresses both endogenous and exogenous estrogen effects.

Our results are important for a number of reasons. First, there are important gender differences in the renal response to ANG II (35), and estrogen produces profound and complex effects on the circulating and renal RAS (49). Estrogen replacement may be beneficial for renal function, delaying the progression of renal disease (16). There is an interaction between estrogen and AT₂ receptors in reproductive organs. Stimulation of AT₂ receptors located in ovarian granulose cells induced ovulation and oocyte maturation, and increased estrogen production (59). In turn, estrogen increased the AT₂ receptor number in human myometrium (29).

Second, AT₂ receptor stimulation is associated with vasodilatation, inhibition of growth, and natriuresis, effects opposite those of renal AT₁ receptors and beneficial for renal function (48). Notwithstanding, the possibility of an influence of estrogen on renal AT₂ receptors was not previously considered.

Our results demonstrate that while AT₁ receptors predominate in control male or female kidneys, estrogen treatment in OVX mice dramatically decreased the AT₁-to-AT₂ receptor ratio, a possibly significant change. A mechanism of cross talk between the two ANG II receptor types was proposed on the basis of studies in cell culture (21), and this hypothesis is supported by functional evidence. Overexpression of AT₂ receptors reduces (31) and AT₂ gene-deletion increases (20) AT₁ receptor-mediated responses. AT₁ receptor expression is upregulated in the absence of AT₂ receptor expression (46, 47), explaining the increased
sensitivity to AT\textsubscript{1} receptor stimulation in this model (20). This indicates that AT\textsubscript{2} receptor expression contributes to control AT\textsubscript{1} receptor expression and function. In addition, AT\textsubscript{1} receptor blockade produces renal vasodilatation (26) and AT\textsubscript{2} receptor stimulation enhances the antihypertensive effects of AT\textsubscript{1} receptor antagonists (3).

Depending on the localization of the receptor types, their cross talk can occur in the same cell or in different cells through indirect mechanisms. AT\textsubscript{1} and AT\textsubscript{2} receptors are colocalized in glomerular capillary loops, particularly in podocytes enveloping glomerular capillaries. This indicates the strong possibility of same-cell cross talk between receptor types. In some other areas of the kidney, autoradiographic techniques did not find indications of a possible colocalization. For example, the renal capsule expressed AT\textsubscript{2} but not AT\textsubscript{1} receptors, and only AT\textsubscript{1} receptor binding was present in cortical structures other than glomeruli. While these findings may indicate that cross talk between receptor types in these structures is only indirect, or that it does not exist, we should also consider our methodological limitations. Although AT\textsubscript{1} and AT\textsubscript{2} receptor detection by immunohistochemistry appears similar, the number of AT\textsubscript{1} receptors in glomeruli is 10 times higher than that of AT\textsubscript{2} receptors when quantitated by autoradiography. It is therefore possible that low levels of receptor protein or receptor binding can escape the limitations of the methods used here.

The altered balance in AT\textsubscript{1}/AT\textsubscript{2} receptor expression produced by estrogen administration may have profound implications because of the opposing effects of AT\textsubscript{1} and AT\textsubscript{2} receptor stimulation (48). Thus a more favorable balance in the direction of AT\textsubscript{2} receptor stimulation could increase flow-induced dilation in resistance arteries (32), improve renal blood flow and enhance pressure natriuresis (15), reduce blood pressure (15) and offer protection from hypertension (5), inhibit cell growth (7) and the development of kidney fibrosis (28), and reduce renal hypersensitivity to ANG II (53). Thus the enhanced AT\textsubscript{2} receptor expression after estrogen treatment could counteract the stimulation by estrogen of renal ANG II levels (6). This could explain the gender differences in the renal response to ANG II, such as the blunting of the decrease in renal plasma flow and the filtration fraction after ANG II administration observed in women (35) and the slower progression of renal disease in women compared with men. Alterations in the balance of renal AT\textsubscript{1} and AT\textsubscript{2} receptor expression can also contribute to the ANG II-medi-

![Figure 4](http://ajprenal.physiology.org/)

**Fig. 4.** Emulsion autoradiography of AT\textsubscript{2} receptor binding in mouse kidney. A, C, E, and G: autoradiographic images from sections obtained from female mice. B, D, F, and H: H&E staining of consecutive sections. White arrows, renal capsule (A and B) and medullary rays (E and F). C and D: glomerulus. G and H: inner medulla (loops of Henle, collecting ducts). Bars, 70 μm.
ated glomerular injury in diabetic nephropathy, whereby AT$_2$ receptor expression is downregulated (57).

We report that estrogen administration to OVX mice increases the content of vasodilatory PGE$_2$ in the inner medulla, an effect related to RAS stimulation (23). AT$_2$ receptor stimulation increases the production of bradykinin, leading to NO release (55) and induction of PGE$_2$ production (30). In addition to vasodilatation, PGE$_2$ inhibits growth of mesangial cells (58) and reduces the expression and secretion of collagen (60). This could be related to the inhibitory effect of estrogen on mesangial cell collagen synthesis, another mechanism postulated in the clinically useful effect of estrogen in ameliorating progressive renal disease (41). Whether the protective effect of estrogen in the kidney is in any way related to AT$_2$ overexpression and to the changes in AT$_1$/AT$_2$ receptor ratio is not known. However, AT$_2$ receptor stimulation has been reported to decrease soluble collagen concentrations (25) and to block the production of renal fibrosis (37). On the other hand, in male rats, the regulation of PGE$_2$ formation under conditions of salt depletion leading to stimulation of renal RAS is increased by AT$_1$ receptors and inhibited by AT$_2$ receptor stimulation (52), indicating complex regulatory mechanisms under different pathophysiological conditions.

Estrogen administration after ovariectomy increases renal medullary endothelial and inducible nitric oxide synthase levels (42), and this may contribute to the higher papillary blood flow and the slower progression of renal disease seen in females (51). The natriuresis, vasodilatation, and reduced blood pressure associated with AT$_2$ receptor stimulation may involve the participation of cGMP, an effect mediated in the kidney by nitric oxide production (55) and in turn dependent on the formation of bradykinin (13, 55). Conversely, ANG II blocks nitric

Fig. 5. Immunocytochemical localization of AT$_1$ and AT$_2$ receptors in the mouse kidney glomerulus. Colocalization of ANG II receptor types in 8-μm-thick sections from the kidney of a female mouse is shown. A: positive AT$_1$ immunostaining (green) in glomeruli. B: positive AT$_2$ immunostaining (red) in glomeruli. C: double AT$_1$/AT$_2$ immunostaining (yellow) in glomeruli. D: AT$_1$ immunostaining (green) localized to glomerular capillary loops. E: AT$_2$ immunostaining (red) localized to glomerular capillary loops. White arrows point to podocytes surrounding glomerular capillaries. Bars, 100 μm.
oxide production by stimulation of AT$_1$ receptors (39). We report decreased cGMP levels in the renal medulla of OVX+E mice, indicating an inhibitory effect of estrogen. However, basal cGMP levels were not increased in OVX mice, suggesting that other ovarian factors contribute to regulate cGMP metabolism. Our results are apparently contradictory to the increased cGMP release into the renal interstitial fluid as a consequence of AT$_2$ receptor stimulation during conditions of sodium restriction in male rats (52). It is possible that decreased cGMP content represents the counterpart of increased release to the renal interstitial tissue. On the other hand, AT$_2$ receptor stimulation reduces basal cGMP levels in adrenal medulla (22), probably as the result of inhibition of guanylate cyclase (4). In addition, other mechanisms have been proposed to explain the vasodilatory effect of AT$_2$ receptor stimulation, such as the activation of a cytochrome P-450 pathway (2). Our results and those in the literature indicate that although a role for AT$_2$ receptors in renal vasodilation appears clear, the precise mechanisms and potential differences related to experimental conditions and the degree of renal RAS stimulation remain open questions.

Estrogen not only upregulates AT$_2$ receptor expression but also increases AT$_2$ receptor mRNA. Through the estrogen receptor (ER), estrogen may activate transcription from the classic hormone response elements (ERE) or from alternative response elements. The promoter region of the AT$_2$ receptor does not contain the consensus ERE (19). However, EREs for different estrogen-responsive genes vary considerably in sequence from that of consensus elements, and the functional ERE in the mouse c-fos gene was identified in the 3'-untranslated region (54). Alternatively, estrogen-liganded ER may regulate AT$_2$ receptor expression at the activator protein (AP)-1 site, because the AP-1 sequence is located in the promoter region of the mouse AT$_2$ receptor (19), and estrogen-liganded ER enhances AP-1 target genes, altering the transcriptional activity of the Jun-Fos complex (24). Additionally, estrogen may affect receptor expression by altering the stability of AT$_2$ mRNA. Clarification of these mechanisms will require additional studies.

In conclusion, we report that estrogen administration in OVX mice profoundly upregulates AT$_2$ receptor expression in the mouse kidney, altering the AT$_1$/AT$_2$ receptor expression ratio. Our results suggest that an altered AT$_1$/AT$_2$ receptor balance may contribute to the protective effects of estrogen in renal disease.

The authors thank Dr. Gustavo Baiardi for help in the preparation of the figures.

J. A. Terrón was on leave from the Department of Pharmacology, Cinvestav-IPN, (Mexico) during this work.
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