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Upregulation of angiotensin II type 2 receptor expression in estrogen-induced pituitary hyperplasia

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Suarez, Cecilia, Graciela Díaz-Torga, Arturo González-Iglesias, Carolina Cristina, and Damasia Becu-Villalobos. Upregulation of angiotensin II type 2 receptor expression in estrogen-induced pituitary hyperplasia. *Am J Physiol Endocrinol Metab* 286: E786–E794, 2004. First published January 13, 2004; 10.1152/ajpendo.00477.2003.—Recent evidence shows that reexpression and upregulation of angiotensin II (ANG II) type 2 (AT2) receptor in adult tissues occur during pathological conditions such as tissue hyperplasia, inflammation, and remodeling. In particular, expression of functional AT2 receptors in the pituitary and their physiological significance and regulation have not been described. In this study, we demonstrate that chronic in vivo estrogen treatment, which induces pituitary hyperplasia, enhances local AT2 expression (measured by Western blot and RT-PCR) concomitantly with downregulation of ANG II type 1 (AT1) receptors. In vivo progesterone treatment of estrogen-induced pituitary hyperplasia did not modify either the ANG II receptor subtype expression pattern or octapeptide-induced and AT1-mediated calcium signaling. Nevertheless, an unexpected potentiation of the ANG II prolactin-releasing effect was observed in this group, and this response was sensitive to both AT1 and AT2 receptor antagonists. These data are the first to document that ANG II can act at the pituitary level through the AT2 receptor subtype and that estrogens display a differential regulation of AT1 and AT2 receptors at this level.

prolactin; calcium signaling; Western blot; reverse transcriptase-polymerase chain reaction

ANGIOTENSIN II (ANG II) is a key regulator of cardiovascular homeostasis and is also involved in various biological functions, such as hormone secretion, tissue growth, and neuronal activation. Two ANG II receptor subtypes, AT1 and AT2, first distinguished on a pharmacological basis, have been identified by expression cloning from various species, and most biological functions exerted by ANG II are mediated by the AT1 receptor subtype. The AT2 receptor subtype is abundantly and widely distributed in fetal tissues, although its expression is dramatically decreased after birth, being restricted to a few organs such as brain, adrenal, heart, myometrium, and ovary (25, 36). Nevertheless, the AT2 receptor is reexpressed in the adult animal under certain pathological conditions, such as ovarian atresia, cardiac and vascular injury, nerve crush, wound healing, and kidney obstruction. AT2 receptors may act as modulators of complex biological programs involved in embryonic development, cell differentiation, tissue protection, and regeneration, as well as in programmed cell death (25, 36).

It has been described that all of the components of the renin-angiotensin system (RAS) are present in the pituitary and that ANG II is produced locally (13). The anterior pituitary predominantly expresses the AT1B isoform of the AT1 receptor (31, 41), with a low expression of the AT1A isoform and the AT2 receptor subtype (41, 45). ANG II releases prolactin both in vivo and in vitro (14) and activates extracellular signal-related kinase 1/2 through a calcium-dependent, AT1 receptor-specific mechanism (47). Yet, participation of AT2 receptors in ANG II effects on pituitary function has not been described to date.

Estrogens can modulate many aspects of the peripheral and pituitary RAS. They reduce the circulating levels of ANG II in ovariectomized rats (9) and significantly decrease the expression of angiotensin-converting enzyme and circulating levels of ANG II in the hypertensive renin-transgenic rat (7). On the other hand, estrogens increase plasma angiotensinogen levels and enhance angiotensinogen mRNA in the liver, brain, and pituitary (23). Besides, ANG II receptor number in the pituitary fluctuates during the estrous cycle, with highest binding in diestrus and lowest in estrus, and chronic estrogen treatment reduces ANG II binding to AT1 receptors in the pituitary (42).

Chronic administration of estrogens to rats induces prolactin-secreting pituitary hyperplasia, and, as we reported in a previous work (15), the response of pituitary cells to ANG II in vitro is modified. We found that intracellular prolactin secretion, Ca^{2+} mobilization, and inositol phosphate generation in response to the octapeptide is altered (19), which can be partly explained by the estrogen-induced decrease of AT1 receptors in pituitary.

In contrast, there are no data concerning the regulation of AT2 receptors by estrogen in the anterior pituitary. Interestingly, it has been shown that estrogen modifies AT2 expression in the myometrium (33). Moreover, both receptors can mutually influence their gene expression profiles (3). Therefore, in view of the observed estrogen-induced downregulation of AT1 receptors in anterior pituitary, we set out to determine whether in vivo estrogen treatment modified the expression and function of AT2 receptors in this tissue. In numerous studies, including gene knockout experiments, the AT2 receptor has been shown to counteract the ANG II effects mediated by the AT1 receptor, suggesting that AT2 might provide a brake for AT1-dependent ANG II signaling (36). Furthermore, the activation and desensitization properties of AT1 and AT2 receptors might also contribute to explain ANG II-induced selective effects in tissues where both receptor subtypes are expressed. In this regard, it has been described that the fate and intracellular

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trafficking of the AT1 and AT2 receptors are different (24), and only the AT1 receptor desensitizes upon prolonged exposure to ANG II, constituting another mechanism by which the octapeptide might terminate AT1-dependent signaling while favoring AT2-coupled pathways.

In addition, we studied the ability of progesterone to counteract the effect of estrogen on the expression profiles of pituitary ANG II receptor subtypes and on ANG II-induced prolactin release. Progesterone can interact in a synergistic or antagonistic manner with estradiol to regulate prolactin secretion. Indeed, the role of progesterone in the regulation of various aspects of reproductive physiology is complex and difficult to evaluate because its effects are modified in extent and direction by the endogenous hormonal milieu (6).

We have therefore examined whether chronic treatment with estrogen alone or in combination with progesterone was able to produce relative changes in ANG II receptor subtype expression in the pituitary gland. We also studied whether the observed changes in receptor expression could be correlated with modifications in ANG II action (prolactin secretion and intracellular Ca^{2+} mobilization) in pituitary cells and their differential response to selective ANG II receptor antagonists.

METHODS

Animals and in vivo treatments. Sixty-day-old female Sprague-Dawley rats were housed in an air-conditioned room with lights on at 0700 and off at 1900. Animals were first divided into two groups, control (sham implantation) and hyperplastic (diethylstilbestrol, DES). Pituitary hyperplasia was induced by subcutaneous implantation of 20 mg of DES (Sigma, St. Louis, MO) for 8 wk. Fifteen days after implantation, both groups were further subdivided into two groups. DES-implanted rats were administered either sesame oil (DES) or 17-OH-progesterone caproate (DES + P4, 5 $mg \cdot wk^{-1} \cdot rat^{-1}$ sc; Schering, Buenos Aires, Argentina) for the next 6 wk. Sham-implanted rats were similarly treated with either sesame oil (controls, CON) or P4 during the same period. Control rats were used in diestrus.

Western blot of AT1 and AT2 receptor. Anterior pituitaries were homogenized (1 mg in 10 μl) in ice-cold buffer containing 1 mM K_2PO_4, 1 mM $MgCl_2$, and 320 mM sucrose, pH 7.4, in a hand-held microtissue homogenizer. The homogenate was then centrifuged at 700 *g* for 10 min at 4°C. The supernatant was removed and further centrifuged for 15 min at 18,000 *g* at 4°C to precipitate the membrane fraction. The pellet was resuspended in the initial volume with Krebs-Henseleit-Tris buffer (in mM: 20 Tris·HCl, 118 NaCl, 1.2 KH_2Po_4, 1.2 $MgSO_4$, 4.7 KCl, 1.8 $CaCl_2$, and 5.6 glucose, pH 7.4), and a 3-μl aliquot was taken to quantify proteins by the Lowry method. Homogenates were then centrifuged for 15 min at 18,000 *g* at 4°C, and the resulting pellets were diluted with sample buffer (62.5 mM Tris·HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 50 mM dithiothreitol, pH 6.8) to give a final protein concentration of 100 μg/20 μl. Samples were heated for 5 min at 95°C and stored at −20°C until use in Western blot analysis.

Membrane extracts (100 μg of protein) from each experimental group were subjected to SDS-polyacrylamide gel electrophoresis. Samples were then blotted onto a nitrocellulose membrane (Bio-Rad, Buenos Aires, Argentina) and probed with the corresponding primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. Polyclonal AT1 and AT2 antibodies of proven specificities (17, 22, 32, 34) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham). For repeated immunoblotting, membranes were incubated in stripping buffer (62.5 mM Tris, 2% SDS, 100 mM mercaptoethanol, pH 6.7) for 40 min at 50°C and reprobed. Band intensities were quantified using the ImageQuant software.

Preparation of pituitary RNA. Total RNA was isolated from anterior pituitaries with TRIzol reagent (GIBCO, Buenos Aires, Argentina). Each gland was homogenized in 500 μl of TRIzol, sonicated for 10 s, and incubated at 30°C for 5 min. Chloroform (80 μl) was added, and after 2 min of incubation, samples were centrifuged at 12,000 *g* for 15 min at 4°C. Isopropanol (200 μl) was added to the supernatant to precipitate the RNA. After a 10-min incubation at 30°C, samples were centrifuged at 12,000 *g* for 10 min at 4°C, their supernatants discarded, and their pellets washed with 400 μl of 70% ethanol. The resulting pellets were finally resuspended in diethyl pyrocarbonate-treated water. RNA was quantified by UV spectrophotometry and its integrity checked by gel electrophoresis.

Semiquantitative RT-PCR for AT1 and AT2 receptor subtypes. Total RNA (2 μg) was reverse transcribed in a reaction mixture containing 10 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, 5 U of RNase inhibitor (Promega, Madison, WI), 1 μg of random hexamers (Biodynamics SRL, Buenos Aires, Argentina), and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Buenos Aires, Argentina) in a final volume of 25 μl. After incubation at 37°C for 60 min, the samples were heated for 10 min at 75°C to terminate the reaction. The reverse-transcribed product was amplified with AT1, AT2, or ribosomal 28S sense and antisense primers in a reaction mixture (50 μl) containing 20 mM Tris·HCl (pH 8.4), 50 mM KCl, $MgCl_2$ (Table 1), 0.2 mM dNTPs, 0.5 μM of each primer, and *Taq* DNA polymerase (Invitrogen Life Technologies; using an Eppendorf thermal cycler).

In Table 1, primer sequences and conditions of PCR amplification are detailed. Common steps were a hot-start step of 3 min at 93°C, followed by *n* cycles of denaturation at 93°C for 60 s, annealing for

Table 1. *Description of primers used and PCR conditions*

	AT1	AT2	Ribosomal 28S
Forward primer sequences (5′ → 3′)	CAA GGC TGG CAG GCA CAA	GCT GTT GTG TTG GCA TTC AT	GCC TAG CAG CCG ACT TAG AA
Reverse primer sequences (5′ → 3′)	GAG GCG AGA CTT CAT TGG GT	TCC AAA CCA TTG CTA GGC T	TTC ACC GTG CCA GAC TAG AG
Nucleotides (forward)	214–231	920–939	7,181–7,200
Nucleotides (reverse)	550–569	1,260–1,278	7,513–7,532
Amplicon length (bp)	355	358	351
Reference no.	29	30	48
$MgCl_2$, mM	1	1	2
cDNA, μl	1.2	12.0	0.6
Taq DNA polymerase, U	1.5	1.5	2.5
Annealing temp, °C	60	55	52
Cycles, no.	35	40	30

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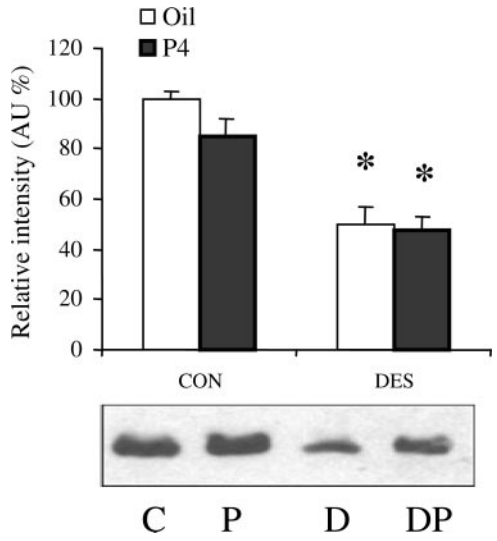

Fig. 1. Densitometric analysis of ANG II type 1 receptor (AT1) Western blots in oil- (open bars) and progesterone (P4; filled bars)-pretreated, control (CON) and diethylstilbestrol (DES)-treated rats. Arbitrary units (AU) of band intensities were normalized to those of the control group in each series of experiments. *$P < 0.05$ vs. respective matched control; $n = 11$, 7, 10, and 11, respectively. For this and following figures, results shown are means ± SE. *Bottom*: representative Western blot. C, control; P, P4; D, DES; DP, DES + P4; molecular mass 45 kDa.

60 s and extension at 72°C for 40 s, with a final elongation step of 5 min at 72°C.

AT1 sense and antisense primers were designed from the homologous sequences of the coding region of the rat AT1A and AT1B receptor genes. Because the coding regions of the rat AT1A and AT1B receptor genes are composed of only one exon, contamination of sample RNAs by genomic DNA was checked by directly subjecting the RNA samples to PCR amplification without an RT step.

Preliminary experiments using various RNA concentrations and cycle numbers confirmed that these PCR reactions were performed within the linear phase of the PCR amplification reaction. Ten microliters of amplified mixture were mixed with 1 μl of sample buffer (25% bromophenol blue, 30% glycerol) and analyzed by 1.8% agarose gel electrophoresis. The amplified DNA bands were detected by ethidium bromide staining. Densitometric analysis was conducted using ImageQuant software, and intensity values of AT1 and AT2 PCR products were normalized to those of corresponding ribosomal 28S mRNA.

Cell culture and prolactin secretion. Anterior pituitary cell culture was performed as previously described (4). Cells were incubated for 30 min in 250 μl of Dulbecco's modified Eagle's medium/F-12 nutrient mixture (DMEM-F12, GIBCO), containing 2.2 g/l $NaCO_3H$ and 0.1% BSA, with no further addition (control) or with 1×10^{-8} M ANG II in the absence or presence of either 1×10^{-6} M DUP-753 (an AT1 receptor-specific antagonist; gift from DuPont Merck, Wilmington, DE) or 1×10^{-6} M PD-123319 (an AT2 receptor-specific antagonist; gift from Parke-Davis, Ann Arbor, MI) in quadruplicate. Samples from culture medium were taken and stored at −20°C until prolactin levels were determined by radioimmunoassay.

Intracellular calcium measurement. Measurements were made as previously described (21) in a suspension of pituitary cells from CON, P4, DES, or DES + P4 experimental groups. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response to ANG II (1×10^{-8} M) in the absence or presence of DUP-753 (1×10^{-6} M) or PD-123319 (1×10^{-6} M) was tested in each group.

Radioimmunoassay. Prolactin was measured by radioimmunoassay using kits generously provided by the National Pituitary Agency and Dr. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Results are expressed in terms of prolactin RP_3. Intra- and interassay coefficients of variation were 7.2 and 12.8%, respectively.

Statistical analyses. Receptor expression by Western blot or RT-PCR was analyzed by two-factor analysis of variance (ANOVA) for the effects of group (sham or DES) and treatment (oil or P4). Hormone secretion in vitro was analyzed by two-factor ANOVA for repeated measures for the effects of group and drug. Anterior pituitary weight and basal prolactin release were analyzed by one-factor ANOVA. $P < 0.05$ was considered significant.

RESULTS

Pituitary weights. Average pituitary weight increased 3.7-fold after 8 wk of estrogen treatment in the DES group. When P4 was administered for the last 6 wk of DES treatment, pituitary weight was not significantly different from that in the DES group. Furthermore, pituitary weight was similar in control and P4-treated rats (average pituitary weights in mg ± SE: CON = 13.7 ± 0.6, P4 = 14.6 ± 0.7, DES = 51.3 ± 3.0, DES + P4 = 51.7 ± 4.5; $n = 15$, 9, 12, and 12, respectively; $P < 0.05$ vs. CON and P4).

Protein expression of AT1 and AT2 receptor subtypes in pituitary membrane fractions. The estimated molecular masses of the AT1 and AT2 receptors were 45 and 41 kDa, respectively. In some experiments, the AT1 receptor-specific antibody also revealed a 57-kDa band.

AT1 receptor expression was significantly decreased in anterior pituitaries of DES-implanted rats whether treated with P4 or oil [DES and DES + P4 vs. CON and P4: $F(1,35) = 55.06$, $P = 0.00012$; Fig. 1]. Interaction between group (sham and DES) and treatment (oil and P4) was not significant [$F(1,35) = 1.27$, $P = 0.27$]. AT1 receptor expression in the P4 group was similar to control levels, even though a slight reduction could be observed.

In contrast, pituitary AT2 receptor followed an opposite expression pattern, as it was significantly increased in both oil- and P4-treated DES-implanted groups compared with their matched controls [DES and DES + P4 vs. CON and P4:

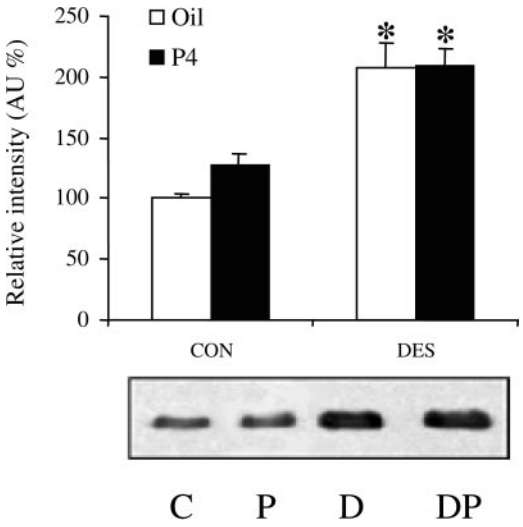

Fig. 2. Densitometric analysis of AT2 Western blots in oil- (open bars) and P4 (filled bars)-pretreated CON and DES rats. AU of band intensities were normalized to those of the control group in each series of experiments. *$P <$ 0.05 vs. matched control; $n = 11$, 7, 10, and 11, respectively. *Bottom*: representative Western blot. Molecular mass 41 kDa.

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$F(1,35) = 64.12$, $P = 0.00012$; Fig. 2]. As in the case of AT1 receptors, interaction between group and treatment was not significant [$F(1,35) = 1.93$, $P = 0.17$], and there were no differences in AT2 receptor levels between the control and P4 groups.

mRNA expression of AT1 and AT2 receptor subtypes. Western blot data were further supported by RT-PCR analysis. Consistently, results indicated that AT1 mRNA levels were significantly lower in DES-implanted groups (DES and DES + P4) compared with sham-implanted groups (CON and P4) [$F(1,34) = 16.58$, $P = 0.00026$]. P4 treatment had no effect in either group, and there was no interaction between group and treatment [$F(1,34) = 0.21$, $P = 0.65$; Fig. 3].

RT-PCR analysis indicated that the AT2 receptor gene was not only transcribed in anterior pituitary but also upregulated in vivo by chronic exposure to estrogen. AT2 mRNA levels were significantly higher in DES-implanted rats (DES and DES + P4) compared with sham-implanted rats [CON and P4: $F(1,26) = 6.36$; $P = 0.018$]. P4 treatment had no effect on AT2 mRNA levels in either group [$F(1,26) = 0.46$, $P = 0.50$], and there was no significant interaction between group and treatment [$F(1,26) = 0.47$, $P = 0.50$; Fig. 4].

ANG II-induced prolactin release in vitro. Basal levels of prolactin release in vitro were reduced ($P < 0.05$) in both DES and DES + P4 groups compared with CON, as previously described (15). In the P4 group, there was an almost twofold increase in basal prolactin release ($P < 0.05$ vs. CON; basal prolactin release in ng/50,000 cells ± SE was as follows: 1,206 ± 99, 1,135 ± 142, 2,968 ± 275, and 4,548 ± 298 for DES, DES + P4, CON, and P4 groups, respectively). Results of ANG II-induced prolactin release were expressed in percentages of these basal levels.

We tested the effect of 30-min incubation with 1×10^{-8} M ANG II, either alone or in combination with AT1- or AT2-specific antagonists on prolactin release. ANG II released prolactin in all experimental groups, but the response was significantly greater in the DES + P4 group compared with the DES and CON groups ($P = 0.00013$ and 0.045, respectively; Fig. 5).

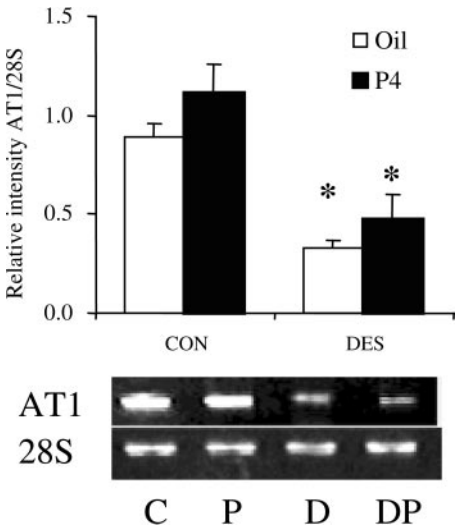

Fig. 3. Densitometric analysis of AT1 RT-PCR products in oil- (open bars) and P4 (filled bars)-pretreated CON and DES rats. For each sample, intensity units of the AT1-specific band were normalized to those of the respective ribosomal (r)28S band. *$P < 0.05$ vs. matched control; $n = 9$, 10, 9, and 8, respectively. *Bottom*: representative RT-PCR of AT1 and r28S transcripts from samples of the different groups.

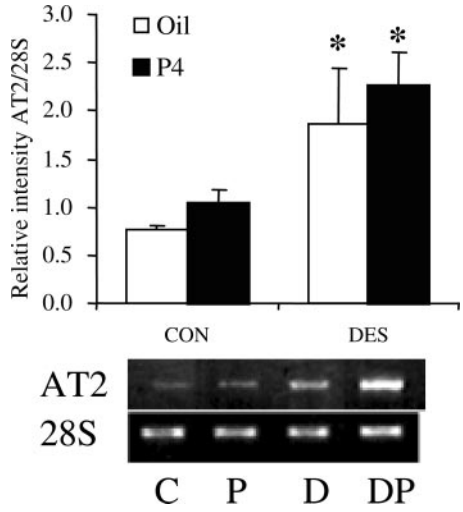

Fig. 4. Densitometric analysis of AT2 RT-PCR products in oil- (open bars) and P4 (filled bars)-pretreated CON and DES rats. For each sample, intensity units of the AT2-specific band were normalized to those of the respective ribosomal 28S band. *$P < 0.05$ vs. matched control; $n = 8$, 7, 7, and 8, respectively. *Bottom*: representative RT-PCR of AT2 and r28S transcripts from samples of the different groups.

Interestingly, a two-factor ANOVA revealed a significant interaction between drug and group [$F(1,54) = 10.63$, $P = 0.00001$]. In both control and DES groups, 1×10^{-6} M DUP-753 (AT1-specific antagonist) blocked ANG II-induced prolactin increase, whereas 1×10^{-6} M PD-123319 (AT2-specific antagonist) did not, indicating that the effect is mediated by the AT1 receptor in these groups (Fig. 5, *A* and *B*). Unexpectedly, in the DES + P4 group, both DUP-753 and PD-123319 blocked the ANG II-induced prolactin release ($P = 0.00012$ and 0.00012, ANG II vs. ANG II + DUP-753 and vs. ANG II + PD-123319, respectively; Fig. 5*C*). These results indicate that both AT1 and AT2 receptors are involved in ANG II-induced prolactin release in the DES + P4 group. DUP-753 and PD-123319 per se did not modify prolactin release in any group (not shown), and the response observed in anterior pituitary cells from the P4 group was not different from CON (Fig. 5*D*).

ANG II-induced $[Ca^{2+}]_i$ mobilization in dispersed cells. In anterior pituitary cells from oil- (CON) and P4-treated sham rats, 1×10^{-8} M ANG II induced a spike response of $[Ca^{2+}]_i$ (Fig. 6*A*). It consisted of a 2- to 6-s delay, followed after 12 ± 2 s by a peak increase of 44.5 and 25.3%, respectively (rise from 257.5 ± 5.5 to a peak of 372.1 ± 11.5 nM for CON and from 280.3 ± 80.3 to 351.2 ± 10.3 nM for P4), and a subsequent decay to resting levels at 50 ± 3 s.

On the other hand, in DES and DES + P4 cells, the spike phase in response to 1×10^{-8} M ANG II was practically absent (Fig. 6*D*), and there was a plateau increase of 20% over basal levels. ANG II increased $[Ca^{2+}]_i$ from 302.5 ± 9.0 nM to a plateau concentration of 363.5 ± 12.4 nM within 21 ± 2 s of stimulation, and levels remained elevated for ≥3 min.

Despite clear differences in ANG II-induced $[Ca^{2+}]_i$ mobilization patterns between control and P4 vs. DES and DES + P4 cells, responses in both groups were blocked by previous

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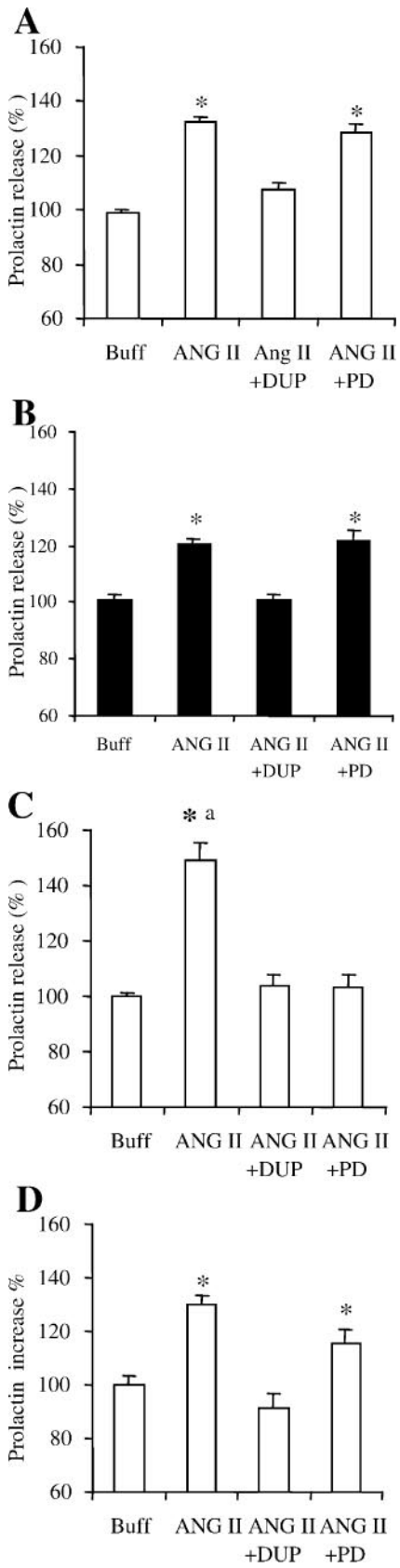

addition of the AT1 receptor-specific antagonist DUP-753 (1×10^{-6} M) (Fig. 6, *B* and *E*), whereas the AT2 receptor-specific antagonist PD-123319 (1×10^{-6} M) was ineffective (Fig. 6, *C* and *F*), indicating that the ANG II-induced intracellular calcium responses were mediated by the same receptor subtype in the different groups.

DISCUSSION

In previous works, we demonstrated that chronic in vivo treatment with estrogens altered the response of rat pituitary cells to ANG II in vitro (15, 19) and lowered total ANG II receptors in the pituitary (42). The aim of this study was to determine the effects of estrogen, and the combined effects of estrogen and progesterone, on the expression of AT2 receptors in the rat pituitary and their possible contribution to the action of ANG II on Ca^{2+} mobilization and prolactin release.

As expected, DES treatment increased pituitary weight, whereas cotreatment of DES rats with progesterone did not modify this situation. In this respect, it has been described that progesterone reduced by 25% the DES-induced pituitary weight increase in female F344 ovariectomized rats (39). Progesterone can modulate estrogen effects on the anterior pituitary, as it is able to antagonize estrogen-induced prolactin secretion and prolactin gene expression (11). In addition, progesterone-mediated inhibition of estrogen proliferative effects has also been shown in the uterus (26), as well as in normal and neoplastic breast tissue (10). The fact that in our experimental design progesterone was not able to reduce or limit the pituitary growth induced by estrogen may be related to differences in estrogen sensitivity between Fischer 344 and Sprague-Dawley rats (53).

Both ANG II receptor subtypes (AT1 and AT2) have structural features of a G protein-coupled, seven-transmembrane domain receptor. The primary sequence of cloned AT1 receptors indicates that its predicted molecular mass for the unglycosylated state is 41 kDa, with greater mass if glycosylation occurs at one or more sites of the five potential sites available (28). Western blot experiments showed that major band sizes for the AT1 receptor corresponded to 57 and 45 kDa. These values are within the range of those found by other workers using similar or different antibodies on different tissues (17, 22, 32, 34, 43, 52). The various sizes of the protein described in the literature no doubt reflect the conditions under which the gels were run, the extent and nature of glycosylation of the protein, and the breakdown products present in the extracts. On the other hand, a single band for the AT2 receptor protein was observed, and it displayed the expected electrophoretic mobility in accord with its theoretical size and with the one described for AT2 receptors in different tissues (17, 32, 35, 37, 43).

The AT1 receptor subtype present in membrane fractions of anterior pituitaries from DES and DES + P4 groups was significantly reduced. This observation agrees with previous

Fig. 5. Effect of ANG II and subtype-specific ANG II receptor antagonists on prolactin secretion in dispersed pituitary cells from CON (*A*), DES (*B*), DES + P4 (*C*), and P4 (*D*) -treated rats. DUP, 1×10^{-6} M DUP-753 (AT1-specific antagonist); PD, 1×10^{-6} M PD-123319 (AT2-specific antagonist); Buff, buffer; ANG II, 1×10^{-8} M ANG II. For each experimental group, results are expressed as percentage of Buff levels. $^{*}P < 0.05$ vs. respective Buff. $^{a}P < 0.05$ vs. ANG II stimulation in control cells; $n = 6$.

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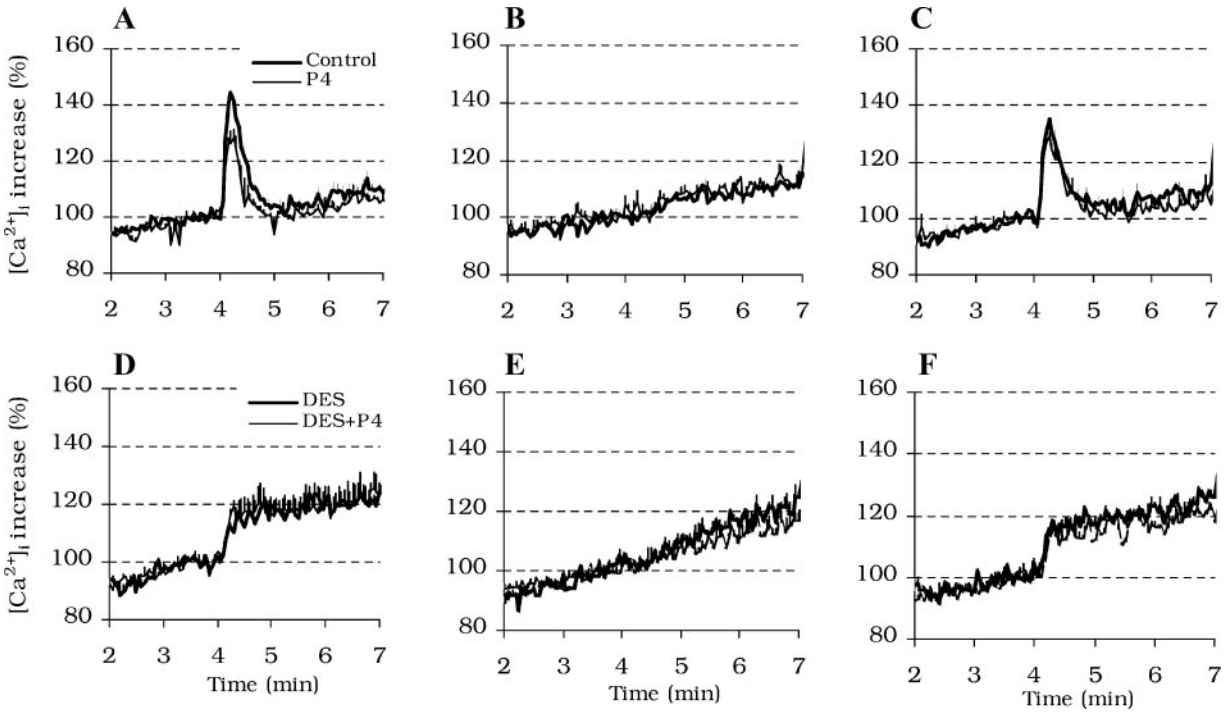

Fig. 6. Effect of ANG II and subtype-specific ANG II receptor antagonists on intracellular Ca^{2+} mobilization in dispersed pituitary cells from oil- and P4-pretreated (*A–C*), or DES- and DES + P4-pretreated (*D–F*) rats. Cells from the different experimental groups were stimulated with 1×10^{-8} M ANG II in the absence (*A* and *D*) or presence of a prior addition of either 1×10^{-6} M DUP-753 (*B* and *E*) or 1×10^{-6} M PD-123319 (*C* and *F*). Curves represent the average of 3 experiments, and in each experiment a batch of cells derived from ≥3 rats/group was used. Results are expressed as %increase of $[Ca^{2+}]_i$ over basal levels (basal levels were considered the average $[Ca^{2+}]_i$ recorded during a 20-s period before the ANG II stimulus). Vertical lines represent SE for each point.

binding and autoradiographic studies (42). Interestingly, Western blot analysis revealed that the AT2 receptor subtype was upregulated in both DES and DES + P4 groups to the same extent. Semiquantitative RT-PCR for the AT2 receptor mRNA required 10 times more template and additional cycle numbers. This is consistent with the low levels of AT2 receptor expression in the pituitary (41, 45). Nevertheless, RT-PCR results support those of Western blotting and also showed increased AT2 mRNA levels (and decreased AT1 mRNA) in the context of in vivo chronic DES exposure.

The fact that the AT1 receptor is downregulated whereas the AT2 is upregulated may have several explanations. Estrogens are known to enhance ANG II biosynthesis by increasing the elaboration of its precursor angiotensinogen. In this regard, in estrogen-treated female rats there was a significant increase in angiotensinogen mRNA in the pituitary (23). ANG II release from the hypothalamus has also been described to rise in response to estrogen and progesterone treatment (38). In turn, in the presence of high local ANG II concentrations, AT1 receptors may undergo homologous desensitization and downregulation in several experimental conditions (21). In fact, and in opposition to AT2 receptors, AT1 receptors seem to be quite sensitive to ANG II-induced downregulation. For example, after treatment with ANG II, AT1 receptors are internalized into intracellular vesicles in human embryonal kidney (HEK) 293 cells stably expressing the ANG II receptor (24), whereas the AT2 receptor subtype does not undergo internalization upon similar stimulation. Besides, in the adrenal of the ovine fetus, an infusion of ANG II decreased AT1 but not AT2 mRNA (52). Similar downregulation of AT1 receptors by ANG II was obtained in ovarian granulosa cells (40), vascular smooth muscle cells (8), and other tissues. Therefore, the decrease in AT1 binding in the pituitary may result from the markedly elevated levels of ANG II in this gland induced by estrogen treatment.

Concerning the AT2 receptor, it is becoming increasingly clear that its expression is upregulated by ANG II and several growth factors in different physiological and pathological processes (5, 16, 51). In addition, it is apparent that there is a reciprocal regulation between receptor subtypes (46, 49), and the direction of the regulation depends on both the cell type and the hormonal milieu to which it is exposed. In several tissues, there is cross talk between AT1 and AT2 receptors, and the expression of AT1 receptors can be dependent on AT2 receptor expression (3, 46). Furthermore, a direct, positive effect of estrogens on the density of AT2 receptors has been demonstrated in the human myometrium (33). Nevertheless, estrogens might upregulate pituitary AT2 by indirect mechanisms, for example by inhibiting AT1-mediated repression of the AT2 receptor gene through downregulation of AT1 receptors. The fact that estrogens can exert a dual regulation on AT1 and AT2 receptors emphasizes the growing appreciation of the complexity and multifaceted nature of estrogen action in reproduction as well as in cardiovascular physiology.

Because ANG II binds to AT1 and AT2 receptor subtypes with a similar affinity, the cellular response should be highly dependent on the relative expression level and/or responsiveness of both receptors, as described in other systems. In this

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context, we sought to determine whether AT2 receptors participate in ANG II-induced intracellular Ca^{2+} mobilization and/or prolactin release in anterior pituitary cells from DES-implanted rats.

As we previously described (15), chronic in vivo treatment with estrogens alters the response of pituitary cells to ANG II in vitro. The pattern of Ca^{2+} mobilization elicited by ANG II is greatly modified; ANG II does not evoke a clear spike elevation of $[Ca^{2+}]_i$ and produces a plateau-type rise in $[Ca^{2+}]_i$ levels. In the present experiments, spike responses were obtained in CON and P4 cells, whereas plateau responses were observed in both DES and DES + P4 cells. But despite the profound differences in the pattern of $[Ca^{2+}]_i$ response to ANG II, only the AT1-specific antagonist blocked ANG II-induced $[Ca^{2+}]_i$ increase in all groups. This indicates that the different intracellular Ca^{2+} signaling patterns induced by ANG II are mediated by the AT1 receptor in the presence and absence of steroids.

As we previously reported for DES-treated cells (20), we found that, when cells from steroid-treated rats were cultured in vitro, basal prolactin release was reduced in cultured cells from DES and DES + P4 groups compared with controls. This effect was probably related to the high exocytotic rate of hyperplastic lactotropes, which become degranulated. A two-fold increase in the basal level of prolactin release was found in the P4 group upon 30 min of stimulation, as has been previously described (50).

Interestingly, our results showed that the prolactin response to ANG II was significantly enhanced in the DES + P4 group. It is clear that in many instances progesterone action requires a background level of estrogen exposure, as progesterone receptor induction is under the influence of estrogen (6). In agreement with our results, it has been described that the combination of estrogen and progesterone favors paradoxical stimulation of prolactin secretion in response to dopamine (12), and the increase in the concentration of thyrotropin-releasing hormone in the pituitary portal plasma and prolactin in arterial plasma (27). So, even though progesterone treatment was not able to counteract DES-induced effects on pituitary weight, expression pattern of pituitary ANG II receptor subtypes and ANG II-induced $[Ca^{2+}]_i$ signaling, it was able to enhance the prolactin-releasing effect of the octapeptide in hyperplastic cells.

It has been described that ANG II-induced prolactin release is mediated by the AT1 receptor (4). But, in the DES + P4 group, both antagonists inhibited the ANG II prolactin release, indicating that in these cells the AT2 receptor was also involved. This is the first demonstration that ANG II can act at the pituitary level through the AT2 receptor subtype, and this finding may be related, in part, to the observed upregulation of AT2 expression. The effect seems to require combined exposure to estrogen and progesterone, as the AT2 antagonist was not effective in cells from DES-only-treated rats. There was no significant difference in receptor expression between the two DES-treated groups that might explain the differential action of the AT2 antagonist on ANG II-induced prolactin release in the DES compared with the DES + P4 group. Subtle differences in receptor number not detected by Western blot or RT-PCR, or indirect mechanisms, may explain the differences encountered. For example, progesterone may upregulate a factor that participates in ANG II-induced AT2-dependent prolactin release, and the increase in both AT2 and P4 receptors produced by DES may be necessary for this action. Finally, this AT2-mediated response appears to be independent of ANG II-induced Ca^{2+} signaling, which is sensitive only to AT1 receptor blockade.

In most instances, AT2 receptors counteract AT1-mediated ANG II actions, but it has also been described that ANG II can have similar effects acting on both receptor subtypes, as in the present study. For example, in rat cardiomyocytes, ANG II-induced apoptosis is mediated through activation of both AT1 and AT2 receptors (18), and in glomerular epithelial cells, both receptor subtypes also participate in ANG II-mediated physiological responses (44).

Even though we did not address the specific cell type localization of the ANG II receptor subtypes within the anterior pituitary, the observed synergy of both receptor subtypes in the ANG II-induced prolactin release supports the hypothesis that AT1 and AT2 receptors are coexpressed in lactotropes. This hypothesis has paramount importance, because it raises the possibility that AT1 and AT2 receptors form heterodimers. In fact, AT1 heterodimerization with AT2 (1) and the bradykinin B2 receptor (2) has been recently shown to either antagonize or enhance AT1-mediated ANG II actions. However, it is also possible that estrogen and/or progesterone, or the hyperplastic process itself, might differentially determine the cell type-specific expression pattern of ANG II receptor subtypes. Indeed, further studies are needed to adequately clarify this issue.

Taken together, these results describe for the first time regulation and function of AT2 receptors in the anterior pituitary. Furthermore, they raise exciting issues regarding the biology of ANG II receptor subtypes and their role in estrogen-induced pituitary adenomas. These studies are of clinical relevance in light of the recent development of antihypertensive treatments using selective AT1 receptor antagonists that promote a significant increase in systemic ANG II levels, which in turn can activate unprecedented biological responses through AT2 receptors. In addition, further understanding of the mechanisms that link estrogen action to the activity of the RAS is relevant to evaluate possible secondary effects of estrogen replacement therapies in postmenopausal women.

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