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Characterization and regulation of the angiotensin II type-1 receptor (binding and mRNA) in human adrenal fasciculata-reticularis cells

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The classical concept of human adrenal physiology indicates that only glomerulosa cells are the target of A-II. Herein, we demonstrated that cultured human adrenal fasciculata-reticularis cells were also responsive to this hormone. Indeed, these cells contained high affinity (K_d = 0.9–1.1 nM) and low capacity (8,000–13,000 sites/cell) A-II receptors, and more than 95% of them were of the type-1. These AT1 receptors are functional since A-II was able to increase cortisol production after 48 h of treatment. These effects were inhibited by losartan, an AT1 antagonist, but not by CGP 42112A, an AT2 antagonist. The expression of the type-1 A-II recptor mRNA was detected in the whole adrenal in both adult and fetus, and in cultured human adrenal fasciculata-reticularis cells. In these cells A-II negatively regulated AT1 receptor mRNA, and this effect was also mediated through the AT1 receptor subtype.

Angiotensin-II type 1 receptor; Human adrenal

1. INTRODUCTION

Angiotensin II (A-II) is a pleiotropic factor which exerts a variety of physiological responses in vascular and non-vascular tissues [1]. Multiple mechanisms of signal transduction have been shown to be involved in A-II actions, including phosphoinositide breakdown, mobilization of intracellular calcium, calcium channels and inhibition of adenylate cyclase activity [2,3].

Two pharmacologically distinct A-II receptor subtypes have been described so far by using specific antagonists [4], but all the effects currently associated with A-II seem to be mediated by the A-II type-1 (AT1) receptors. These receptors have high affinities for the non-peptide antagonist DuP 753/losartan [4,5]. The role of the AT2 receptors, which have high affinities for the peptide CGP 42112A and non-peptide PD 123177 antagonists [6,7], is not well known. Recently, it has been suggested that A-II decreases the cellular concentration of cGMP in neurons through AT2 receptors [8], and Bottari et al. [9] have suggested that AT2 receptors could activate a membrane-associated phosphotyrosine phosphatase. A third type of A-II receptors (AT3) has been postulated [10], but they are not yet well characterized. The gene and/or cDNA encoding for the AT1 receptor has been cloned in the rat [11], bovine [12] and, more recently, in the human [13–15].

It has been shown that cultured bovine adrenal fasciculata cells contain high affinity A-II receptors which are coupled to intracellular effectors and are able to increase glucocorticoid production [16–18]. In contrast, it has been suggested that human adrenal fasciculata cells do not respond to A-II [19]. In this paper, we have attempted to study the A-II receptors in cultured human adrenal fasciculata cells and we have demonstrated that adult human adrenal fasciculata-reticularis cells contain functional AT1 receptors and corresponding mRNA which are negatively regulated by the hormone.

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2. MATERIALS AND METHODS

Synthetic angiotensin-II (A-II) was purchased from Bachem (Bubendorf, Switzerland); insulin, transferrin and bacitracin from Sigma (Saint-Louis, MO, USA), bovine serum albumin (BSA) from Boehringer (Meylan, France); nystatin from Gibco (Paris, France); penicillin/streptomycin, fetal calf serum and Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F-12 (DMEM/F12 1:1) from J-Bio (Villejust, France). Specific antagonist DuP-753 or losartan was obtained from DuPont Merck (Wilmington, USA) and CGP42112A was a gift of Dr. de Gasparo (Ciba-Geigy, Basel, Switzerland).

2.1. *Isolation, subcloning and expression of the human angiotensin-II type 1 receptor*

A human liver λgt11 cDNA library (Clontech, Palo Alto, CA, USA) was screened using PCR methodology. The primers used were synthetic oligonucleotides specific for the λgt11 phage sequence and synthetic oligonucleotides specific for the published bovine AT1 receptor sequence [12] at first, or specific for the human sequence as soon as we determined it. PCR fragments obtained were sequenced by dideoxy chain termination [20] using [α-^{35}S]dATP and Sequenase (US Biochemical Corp., Cleveland, OH, USA) according to the instructions provided and after subcloning in *Sma*I-cleaved ptZ18 (Pharmacia-France, St. Quentin en Yvelines). The nucleotide sequence for human AT1 receptor obtained was identical to that recently published [13].

We then subcloned a fragment containing the whole translated sequence of the human AT1 receptor into the *Eco*RI/*Bam*HI-cleaved

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eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA, USA), giving pSG5-hAT1. This fragment was recovered from the recombinant ptZ18 vector after cutting by the *Eco*RI and *Bam*HI enzymes. Studies of A-II binding were done after transfection of COS-7 cells using the DEAE-dextran method, as previously described [21].

2.2. *Human adrenal cell isolation and culture*

Human adult adrenals were obtained after organ removal for transplantation from brain-dead patients, with the approval of the ethical committee of the Hospices Civils de Lyon. After having removed the major part of the medulla, the zona fasciculata-reticularis was separated from the capsula and used for cell preparation. Cells were dispersed by two collagenase digestions (1 mg/ml) in DMEM/F-12 medium supplemented with NaHCO3 (14 mM) and HEPES (10 mM) and containing gentamycin (20 μg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and nystatin (100 U/ml). Cells were then placed in monolayer cultures in the same medium containing 2% fetal calf serum plus insulin (10 μg/ml), transferrin (10 μg/ml) and vitamin C (10^{-4} M). After 24 h, the medium was changed to the same medium without serum. Treatments were conducted in this defined medium starting on day 3 of culture.

2.3. *Receptor binding assays*

A-II and the AT2 receptor antagonist CGP 42112A were labeled by the iodogen method [22]. The method used for [^{125}I]A-II purification has been described [22] and [^{125}I]CGP 42112A was purified by HPLC on a C18 μBondapak column (Millipore, Guyancourt, France). The receptor assay was performed on COS-7 cells 60–65 h after transfection with pSG5-hAT1, or at day 3 or 4 of culture for the human adrenocortical cells. According to the procedure described by Crozat et al. [16], binding was carried out in 0.5 ml F12-DME medium containing 0.1% bacitracin, 0.5% BSA and about 2×10^5 cpm (0.2–0.3 nM) of labeled A-II (or labeled CGP 42112A) for 1 h at 37°C. Non-specific binding was determined by the addition of 10^{-6} M unlabeled A-II (or unlabeled CGP 42112A). Competition experiments were performed in the presence of various concentrations of A-II, or in the presence of the specific antagonists losartan and CGP 42112A. At the end of the incubation, binding medium was removed and cells were washed 3 times with cold NaCl 0.9%. The cells were recovered after lysis with 0.4% deoxycholate in 0.5 M NaOH.

2.4. *RNA extraction and Northern blot analysis*

Total RNA was isolated from tissues or cells by the method of Chomczynski and Sacchi [23]. Then samples were separated by electrophoresis in a 1.0% agarose gel containing 8% formaldehyde. RNA was then transferred to a Hybond-N membrane (Amersham, Les Ulis, France). Prehybridization was performed in sodium citrate buffer (SSC 5 ×) pH 7.0, containing 50% formamide, 1 × PE (5 × PE = 250 mM Tris-HCl, pH 7.5; 0.5% sodium pyrophosphate; 5% SDS; 1% polyvinylpyrrolidone; 1% ficoll; 25 mM EDTA and 1% BSA), and 150 μg/ml salmon sperm DNA at 42°C for 4 h. Hybridizations were performed in the same buffer at 42°C for 24 h, using a 918-bp fragment of the human AT1 receptor as a probe. Labeling of this probe in the presence of [α-^{32}P]dCTP was performed with a Multiprime DNA labeling system (Amersham). The blots were washed 2 times in a 2 × SSC buffer containing 0.1% SDS at room temperature for 15 min; one time in 0.2 × SSC containing 0.1% SDS at room temperature for 30 min, and finally in 0.2 × SSC plus 0.1% SDS at 55°C for 5–10 min. Radioactivity was quantified by scanning densitometry using a Preference HIT (Sebia, Paris, France).

3. RESULTS AND DISCUSSION

Displacement of labeled A-II by unlabeled hormone was performed on cultured human adrenal fasciculata-reticularis cells (HAC) (Fig. 1). Scatchard analysis (Fig. 1B) indicated the presence of 8,000 to 13,000 sites/cell, which represents a low number when compared to those found in bovine or ovine adrenocortical cells which have close to 100,000 sites per cell [16,24]. The apparent K_d (0.9–1.1 nM) was found to be the same order of magnitude as that for AT1-transfected COS cells (2.9 ± 1.6 nM) (Fig. 2), which is consistent with values obtained by other authors [13,14]. The majority of A-II binding to HAC was displaced to 1.5 ± 2.2% of control by the addition of losartan (an AT1 specific ligand) at 10^{-5} M (n = 7). When the binding assay was done in the

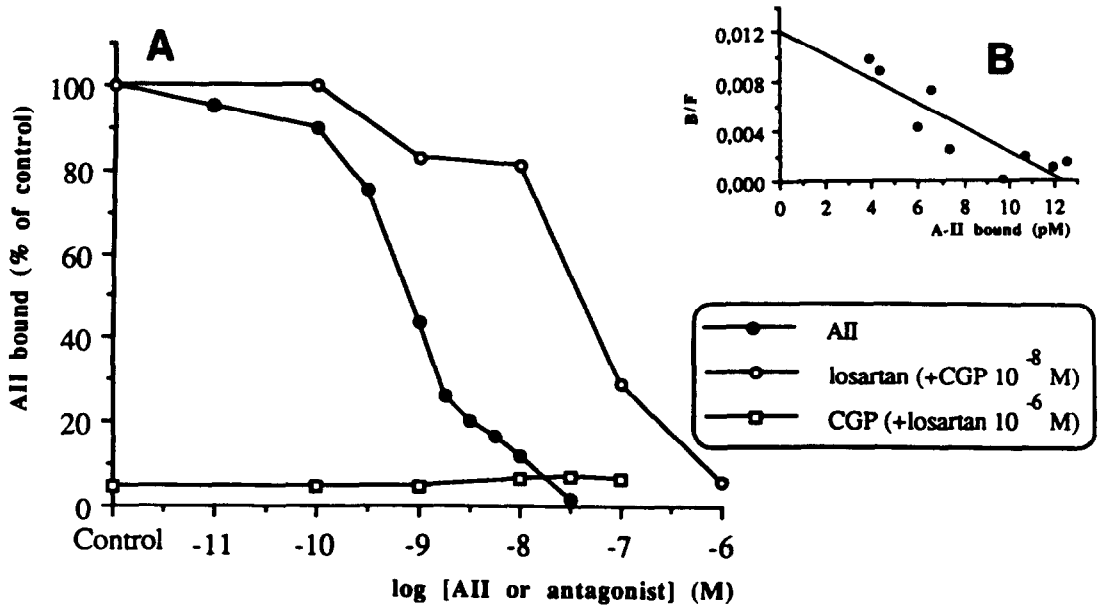

Fig. 1. (A) Displacement of [^{125}I]A-II bound to HAC by increasing concentrations of unlabeled A-II, of losartan in the presence of 10^{-8} M CGP 42112A or of CGP 42112A in the presence of 10^{-6} M losartan. B (inset) shows the Scatchard plot of the binding data for A-II.

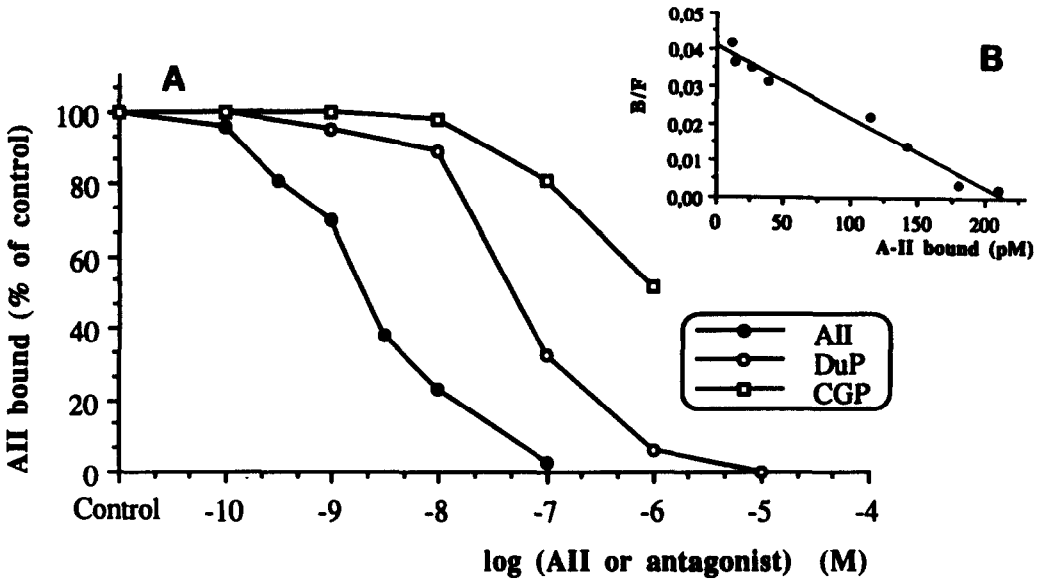

Fig. 2. (A) Displacement of [^{125}I]A-II bound to COS-7 cells transfected with the human AT1 receptor, as described in section 2, by increasing concentrations of unlabeled A-II, DuP 753 (losartan) or CGP 42112A (data represent the means of four different experiments). B (inset) shows the Scatchard plot of the binding data for A-II (K_d = 2.9 ± 1.6 nM with about 135,000 sites/cell).

presence of 5×10^{-8} M CGP 42112A (an AT2 specific ligand), the binding was only inhibited to 93.6 ± 12.6% (n = 6) of the control. These concentrations of losartan and CGP 42112A were chosen because they have been demonstrated to be saturating for the AT1 and AT2 sites in bovine adrenocortical cells (BAC), respectively [18]. In COS cells transfected with pSG5-hAT1, which then expressed only the AT1 receptors, an almost complete displacement was obtained in the presence of 10^{-5} M of losartan with an IC_{50} of about 5×10^{-8} M (Fig. 2). In contrast, CGP 42112A displaced bound A-II only at concentration higher than 10^{-7} M (Fig. 2), concentration at which this compound recognizes AT1 receptor subtype. From these results it seems that only a very small part of the A-II receptors are of the AT2 subtype in HAC (less than 5%). This was confirmed by a second series of experiments where the displacement of labeled A-II by CGP 42112A was performed in the presence of saturating concentrations of losartan (10^{-6} M), while that by losartan was performed in the presence of saturating concentrations of CGP 42112A (10^{-8} M) (Fig. 1A). Residual specific binding was about 5–6% in the presence of 10^{-6} M losartan with no further displacement by CGP 42112A, both in HAC cells (Fig. 1A) and in AT1 transfected COS cells (data not shown).

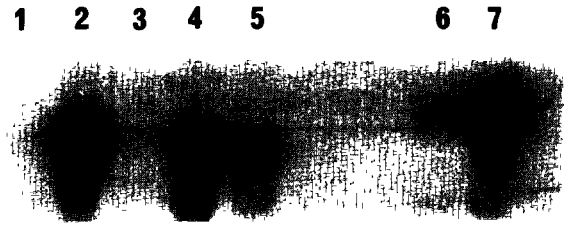

Fig. 3. 30 μg of total RNA from human testis (lane 1), human liver (lane 2), HepG2 cells (lane 3), human adult (lane 4) and fetal (lane 5) adrenal were used in the Northern blot analysis. Electrophoresis also included RNA from bovine liver (lane 6) and adrenal (lane 7). Blot was hybridized with the AT1 receptor probe as described in section 2.

The absence of AT2 receptor subtype in HAC was confirmed by binding studies using labeled CGP 42112A. No specific binding could be demonstrated in either HAC or in AT1 transfected COS cells, when these cells were incubated in the presence of 2×10^5 cpm of [^{125}I]CGP 42112. However, under similar experimental conditions BAC specifically bound about 20% of the total radioactivity, which was displaced by increasing concentrations of unlabeled CGP 42112A, but not by losartan (R. Ouali and J.M. Saez, unpublished results).

Further evidence of the presence of AT1 receptor subtype in HAC was obtained by Northern blot analysis. In human tissues AT1 mRNA was expressed as a single transcript of 2.4 kb, while a single band of 3.3 kb was observed in bovine tissue (Fig. 3). In human the AT1 mRNA levels were higher in liver than in human or fetal adrenal, while in bovine the adrenal content was higher than the liver content. A single AT1 receptor mRNA transcript of 2.4 kb was also observed in HAC (Fig. 4). Treatment of HAC with A-II reduced in a time-dependent manner the amounts of AT1 mRNA, and this effect was abolished by losartan but not by CGP 42112A (Fig. 3). In contrast a four day treatment with ACTH did not significantly modify the AT1 recep-

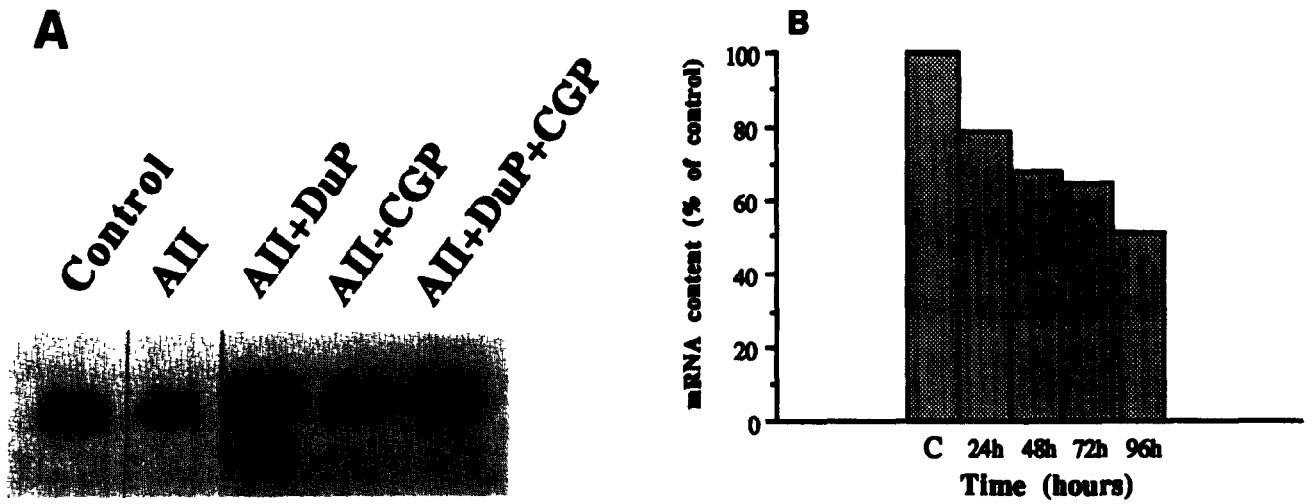

Fig. 4. HAC cells were cultured for 96 h (A) or for the indicated time (B) in the presence of 10^{-7} M A-II with or without losartan (10^{-6} M) and/or 10^{-8} M CGP. Electrophoresis was performed using 15 μg of total RNA and the AT1 receptor cDNA was used as a probe as described in section 2.

tor mRNA levels (data not shown). The inhibitory effects of A-II on AT1 receptor mRNA has also been observed in cultured rat glomerulosa mesangial cells [25] and in BAC (R. Ouali and J.M. Saez, unpublished results).

Next, we investigated whether A-II was able to stimulate the specific function of HAC, namely the secretion of cortisol. Cells were incubated for 24 h without or with ACTH or A-II, and the amounts of cortisol and aldosterone measured in the medium. ACTH produced a 40-fold increase in cortisol secretion, whereas the effects of A-II were less pronounced (5-fold stimulation) (Table I). These effects of A-II were abolished by losartan, but not by the AT2 antagonist CGP 42112A. Moreover, both hormones stimulated about sixfold the secretion of aldosterone. However, the ratio cortisol/aldosterone indicated that the contamination of our preparation with glomerulosa cells was very small. In order to investigate the effects of the pretreatment on the steroidogenic responsiveness, the cells were washed and then incubated 2 h with either ACTH or A-II. In not pretreated cells, both hormones enhanced cortisol production, but the effect of ACTH was higher than that of A-II. Pretreatment with ACTH increased the steroidogenic response to both hormones. This effect is probably related to the positive effect of the hormone on its own receptor [26] and on the expression of the genes encoding for the steroidogenic enzymes [27]. A-II also enhanced the cortisol secretion in response to further stimulation with either ACTH or A-II. Since long-term treatment of zona glomerulosa cells with A-II is unable to shift steroid biosynthesis from an aldosterone-producing to a cortisol-producing pathway [28], the most likely explanations for the above effects of A-II are its positive action on the expression of the ACTH receptor gene and on some of the genes encoding for the steroidogenic enzymes including cytochrome P-450 17α-hydroxylase (M.C. Lebrethon, D. Naville and J.M. Saez, submitted for publication). Moreover, the negative effect of A-II on its own receptors might explain that the responsiveness to A-II of cells pretreated with this hormone was lower than that of ACTH pretreated cells (Table I).

Table I

Pretreatment	ng/mg of protein/24 h		Cortisol ng/2 h	
	Cortisol	Aldosterone	ACTH (10^{-8} M)	A-II (10^{-7} M)
None	47 ± 2	0.10 ± 0.01	97 ± 5	18 ± 1
ACTH (10^{-8} M)	1,971 ± 114	0.63 ± 0.1	452 ± 25	166 ± 9
A-II (10^{-7} M)	249 ± 20	0.77 ± 0.2	201 ± 20	30 ± 2
A-II + DuP (10^{-6} M)	78 ± 8	ND	ND	ND
A-II + CGP (5×10^{-8} M)	274 ± 30	ND	ND	ND

ND, not determined.

Human adrenal fasciculata cells were incubated for 24 h in the presence or absence of the indicated hormones and the cortisol and aldosterone in the medium were measured. Then the cells were washed and incubated in fresh medium containing 10^{-8} M ACTH or 10^{-7} M A-II and after 2 h the cortisol was measured in the medium. The results are means ± S.D. of triplicate determinations of four different wells.

In conclusion, the present results demonstrate for the first time the presence of functional A-II receptors (mainly of the AT1 form) in human adrenal fasciculata-reticularis cells which allow an increase in the production of cortisol and that A-II negatively regulates AT1 receptor mRNA through the AT1 receptors.

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