Original Article

Pathophysiological Roles of the Adrenal Renin-Angiotensin System in Patients with Primary Aldosteronism

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The mechanism of overproduction of aldosterone in primary aldosteronism is unclear. The intraadrenal renin-angiotensin system (RAS) has been suggested to possess the functional role of the synthesizing aldosterone and regulating blood pressure. In order to clarify the pathophysiological roles of adrenal RAS in aldosterone-producing adenoma (APA), we studied the expressions of the messenger RNAs (mRNAs) of renin, angiotensinogen, type 1 (AT1R) and type 2 angiotensin II receptor (AT2R), CYP11B1 (11β-hydroxylase gene) and CYP11B2 (aldosterone synthase gene) in 8 patients with angiotensin II-responsive (ATII-R) APA and compared them with the expressions of the same mRNAs in 8 patients with angiotensin II-unresponsive (ATII-U) APA. Quantification of the mRNA of each gene was done using a real-time polymerase chain reaction with specific primers. There were no significant differences between ATII-R APA and ATII-U APA in the mRNA levels of renin, angiotensinogen, AT1R, CYP11B1 and CYP11B2. The amount of AT2R mRNA was significantly higher in the patients with ATII-R APA than in those with ATII-U APA ($p<0.05$). These results may suggest that AT2R partially contributes to the overproduction of aldosterone in ATII-R APA. (*Hypertens Res* 2006; 29: 9–14)

***Key Words*: aldosterone-producing adenoma, CYP11B2, angiotensin II receptor, renin-angiotensin system**

Introduction

The systemic renin-angiotensin system (RAS) plays an important role in the control of blood pressure and the water electrolyte balance. In the adrenal cortex, it has been shown that renin, angiotensinogen, and type 1 (AT1R) and type 2 angiotensin II receptor (AT2R) are expressed, and together compose a local adrenal RAS that is suggested to have a local physiological function (*1*).

Primary aldosteronism (PA) is defined as hypertension associated with an elevated plasma aldosterone concentration (p-aldo) or elevated secretion rate or urinary excretion of aldosterone in combination with relative suppression of plasma renin concentration or activity (PRA), and the most common clinical subtypes of PA are aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA). PA is much more common than previously thought, based on a systematic screening in referred hypertensive patients (*2*, *3*), and is associated with a greater number of cardiovascular and renal diseases than essential hypertension (*4*, *5*).

Patients with an APA characteristically fail to show a p-aldo increase in response to physiological maneuvers that increase angiotensin II (ATII). Although the aldosterone unresponsiveness in classical ATII-unresponsive (ATII-U) APA has been postulated to be the result of the low density of ATII receptors

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Table 1. Sequences of Oligonucleotide Primers and TaqMan Probes for the Detection of Renin, Angiotensinogen, CYP11B1, CYP11B2 by Real-Time PCR

Target gene		Sequence
Renin	Forward	5′-GGGCATGGGCTTCATTGA-3′
	Reverse	5′-TGGGAGATGATGTTGTCGAAGA-3′
	TaqMan probe	5′-6-FAM-AGGCCATTGGCAGGGTCACCC-TAMRA-3′
Angiotensinogen	Forward	5′-TCTCCCCGGACCATCCA-3′
	Reverse	5′-TGCTCAATTTTTGCAGGTTCAG-3′
	TaqMan probe	5′-6-FAM-CCATGTCCCCAACTGGTGCTGC-TAMRA-3′
CYP11B1	Forward	5′-GGCAGAGGCAGAGATGCTG-3′
	Reverse	5′-TCTTGGGTTAGTGTCTCCACCTG-3′
	TaqMan probe	5′-6-FAM-TGCTGCACCATGTGCTGAAACACCT-TAMRA-3′
CYP11B2	Forward	5′-GGCAGAGGCAGAGATGCTG-3′
	Reverse	5′-CTTGAGTTAGTGTCTCCACCAGGA-3′
	TaqMan probe	5′-6-FAM-CTGCACCACGTGCTGAAGCACT-TAMRA-3′

PCR, polymerase chain reaction.

(*6*), a postreceptor defect (*7*), or the difference between the histological findings and expression of the AT1R messenger RNA (mRNA) (*8*), mechanisms for the paradoxical aldosterone responsiveness in ATII-responsive (ATII-R) APA remain to be elucidated.

ATII stimulates aldosterone synthesis and secretion through AT1R in the adrenal cortex. AT2R are believed to play an important role in cell proliferation and growth, because of their predominant and widespread distribution in fetal tissues. Although the expression of AT2R has also been demonstrated in human adrenal tissue (*9*), the functional role of AT2R in the adrenal glands is unclear. In the present study, in order to clarify the pathophysiological roles of adrenal RAS in APA, we measured the expression of the mRNAs of renin, angiotensinogen, AT1R, AT2R, CYP11B1 (11β-hydroxylase gene) and CYP11B2 (aldosterone synthase gene) in 8 patients with ATII-R APA and compared them with those in 8 patients with ATII-U APA.

Methods

Subjects

We examined 16 patients with APA admitted to our department or associated hospitals. Patients with APA had hypertension (systolic blood pressure: 165.4±6.2 mmHg; diastolic blood pressure: 100.5±3.0 mmHg), low serum potassium concentrations (2.7±0.1 mmol/l), elevated p-aldo (958.1±105.8 pmol/l), and suppressed PRA (0.49±0.16 ng/ml/h). Glucocorticoid-remediable hyperaldosteronism (GRA) associated with adrenal tumor was excluded by chimeric gene analysis as previously described (*9*). A high concentration of plasma aldosterone in the lateral adrenal vein and the presence of a solitary adenoma on CT scan of the adrenal glands confirmed the diagnosis of APA. As controls, normal adrenal cortex adjacent to clinically nonfunctioning adenomas ($n=3$), and normal adrenal cortex obtained during surgical removal for renal cell carcinomas and adrenal cysts ($n=2$) were used.

The study protocol was approved by the Human Research Committee of the Kanazawa University School of Medicine, and all subjects provided their informed consent.

Physiological Maneuvers to Characterize Aldosterone Responsiveness to ATII in Patients with APA

Postural Stimulation Test

In 12 of the 16 patients with APAs, a postural stimulation test was performed in the morning after resting while patients were on regular sodium diets. Briefly, blood was sampled after an at least 30 min rest in the supine position, followed by a second blood sampling taken after standing for 4 h.

Furosemide Administration and Standing Test

In 13 of the 16 patients with APAs, a furosemide administration and standing test was performed in the morning after resting while patients were on regular sodium diets. Briefly, blood was sampled after an at least 30 min rest in the supine position, followed by a second blood sampling obtained after administration of 40 mg furosemide followed by 2 h of standing.

The criteria for the characterization of the aldosterone responsiveness of APA to ATII were as follows: ATII-R APA was defined as an increase in p-aldo to more than the baseline value after 4 h of upright posture or after administration of 40 mg furosemide followed by standing for 2 h, whereas ATII-U APA was defined as a decrease in p-aldo after physiological maneuvers to increase ATII.

Quantification of the mRNAs of Renin, Angiotensinogen, AT1R, AT2R, CYP11B1 and CYP11B2

Adrenal samples were collected after obtaining informed con-

Table 2. The Clinical and Hormonal Data of ATII-R APA and ATII-U APA

	ATII-R APA (n=8)	ATII-U APA (n=8)
Age (years)	45.1±4.5	46.8±4.5
Male/female	3/5	3/5
Systolic BP (mmHg)	164.0±9.7	167.0±8.2
Diastolic BP (mmHg)	99.5±4.3	101.6±4.4
s-K (mmol/l)	2.8±0.1	2.6±0.3
PRA (ng/ml/h)	0.65±0.30	0.30±0.05
p-aldo (pmol/ml)	1,041.2±183.4	875.0±108.9

Data are the mean±SEM. ATII-R APA, angiotensin II-responsive aldosterone-producing adenoma; ATII-U APA, angiotensin II-unresponsive aldosterone-producing adenoma; BP, blood pressure; s-K, serum potassium concentration; PRA, plasma renin activity; p-aldo, plasma aldosterone concentration.

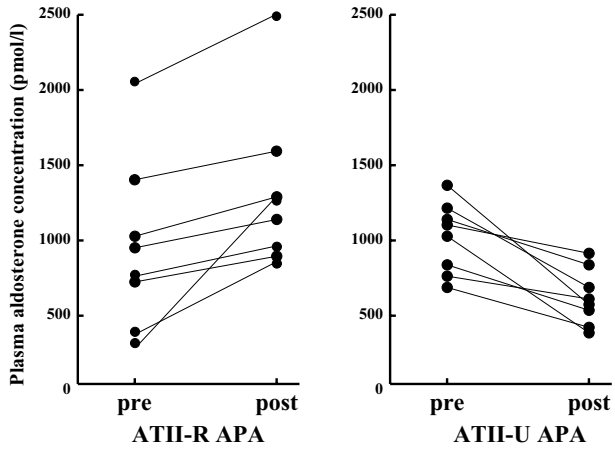

Fig. 1. *Plasma aldosterone concentrations (p-aldo) in the upright test or upright with furosemide administration test. Eight patients were categorized as ATII-R APA because of an increase of p-aldo, and the other 8 patients were categorized as ATII-U APA because of a decrease of p-aldo after the 4-h postural stimulation test and/or the furosemide plus upright posture test. ATII-R APA, angiotensin II-responsive aldosterone-producing adenoma; ATII-U APA, angiotensin II-unresponsive aldosterone-producing adenoma.*

sent and stored at −80°C immediately after tumor resection until extraction of total RNA. Total RNA was isolated from the aldosteronoma tissues and was amplified by a reverse-transcribed polymerase chain reaction (RT-PCR) as previously described (*10*). Briefly, 500 ng of total RNA was incubated at 42°C for 60 min with Superscript II RNase H-transcriptase (Invitrogen Corporation, Inc., Carlsbad, USA) in a 20 μl reaction volume containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l of each dNTP, and 2.5 mmol/l random hexanucleotide primer (PE Applied Biosystems Japan, Tokyo, Japan). Single-stranded cDNA was used for the real-time polymerase chain reaction (PCR). Transcript level quantifications for renin, angiotensinogen, AT1R, AT2R, CYP11B1 and CYP11B2 were performed with real-time PCR primer-probe sets using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems Japan) in a 50 μl reaction mixture containing 1–5 μl (25–75 ng) cDNA template, 25 μl TaqMan Universal PCR master Mix (PE Applied Biosystems Japan), 0.1 μmol/l probe and 0.1 μmol/l of each primer.

The primers and TaqMan probes for each gene are shown in Table 1. For AT1R and AT2R, Assays-on-Demand Gene Expression Products (PE Applied Biosystems Japan) were used as the primers and TaqMan probes. The PCR conditions were established as follows: after incubation at 56°C for 2 min and denaturing at 95°C for 10 min, 45–50 cycles of 95°C for 15 s and 60°C for 1 min were performed. To minimize the effects of variations in deoxyribonuclease digestion and reverse transcription (RT) between samples, mRNA values were normalized as a ratio to the mRNA expression of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, in each sample. GAPDH primers/probes (PE Applied Biosystems Japan) were included in a multiplex with the probes/primers for the genes of renin, angiotensinogen, CYP11B1 and CYP11B2, in single-plex reactions with AT1R and AT2R. The values of each mRNA expression were calculated as a relative fold change in mRNA compared to a control sample. Negative controls contained water instead of first-strand cDNA.

Western Blot Analysis

Tissue extracts (20 μg protein) were separated by SDS-PAGE on 12% (w/v) polyacrylamide gels and electrotransferred to PVDF membranes using a Trans-blot unit (Bio-Rad Laboratories, Tokyo, Japan) for 1.5 h at 100 V. Membranes were blocked with 3% (w/v) BSA in PBS (pH 7.4) containing 0.1% (v/v) Tween 20 (PBST) overnight at 4°C. The membranes were then incubated for 1 h with primary antibody at 24°C. The antibodies used were as follows: anti-AT1R (sc-579, Santa Cruz Biotechnology, Santa Cruz, USA); anti-AT2R (sc-7421, Santa Cruz Biotechnology); anti-β-actin (A5316, Sigma Japan, Tokyo, Japan). After incubation with secondary antibodies, the signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Signals on Western blots were quantified by densitometry and corrected for β-actin.

Statistical Analysis

Data are expressed as the means±SEM. The Mann-Whitney U test was used to evaluate differences in mRNA levels among the three adrenal tissue groups. A p value of less than 0.05 was considered statistically significant.

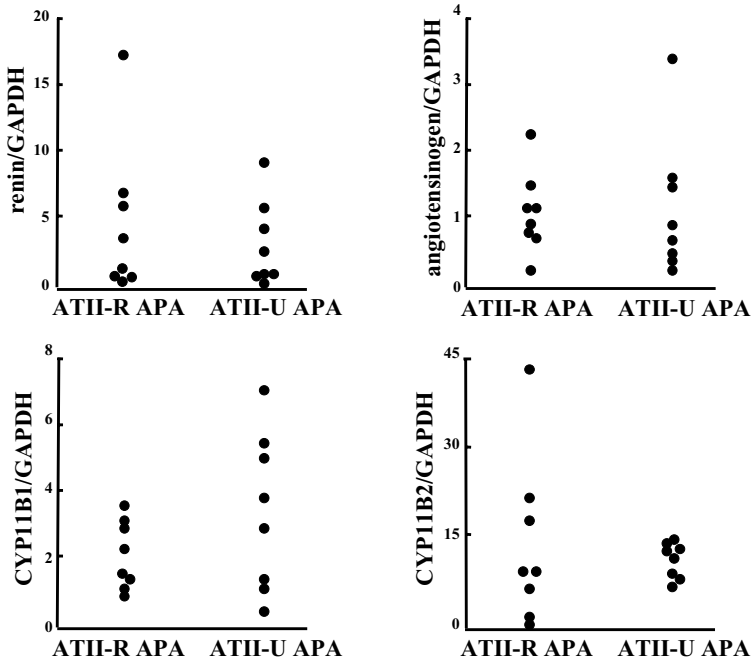

Fig. 2. *There were no significant differences in the levels of the mRNAs for renin, angiotensinogen, CYP11B1, or CYP11B2 between the ATII-R APA and ATII-U APA. GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; ATII-R APA, angiotensin II-responsive aldosterone-producing adenoma; ATII-U APA, angiotensin II-unresponsive aldosterone-producing adenoma.*

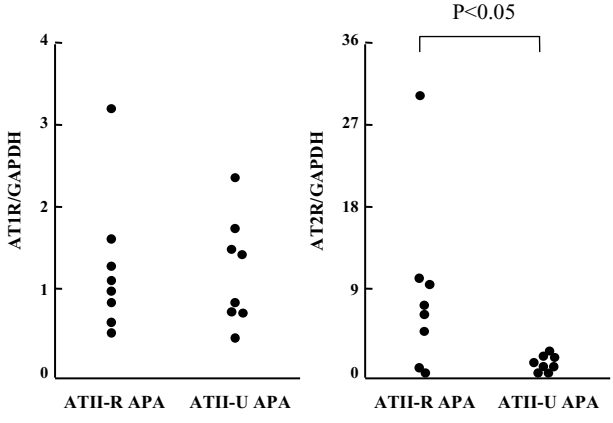

Fig. 3. *The expression levels of the AT1R mRNA were not increased, but the expression levels of the AT2R mRNA were significantly increased in ATII-R APA compared with those in ATII-U APA. GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; AT1R, type 1 angiotensin II receptor; AT2R, type 2 angiotensin II receptor: ATII-R APA, angiotensin II-responsive aldosterone-producing adenoma; ATII-U APA, angiotensin II-unresponsive aldosterone-producing adenoma.*

Results

The clinical and hormonal data of patients with ATII-R APA and ATII-U APA are shown in Table 2. Blood pressure, serum potassium concentrations, PRA and p-aldo did not differ significantly between the subjects with ATII-R APA and ATII-U APA.

Figure 1 shows the data of p-aldo in the upright test or upright with furosemide administration test. The cases were separated into two subgroups, *i.e.*, an aldosterone-increase (8 cases) and an aldosterone-decrease (8 cases) group.

Figure 2 shows the data on the mRNA levels of renin, angiotensinogen, CYP11B1 and CYP11B2 in patients with ATII-R APA and ATII-U APA. There were no significant differences among the subjects with ATII-R APA, those with ATII-U APA, and the normal controls in the mRNA levels of renin (4.5±2.1, 3.0±1.2, 6.2±4.5, respectively), angiotensinogen (1.0±0.2, 1.1±0.4, 1.7±0.6, respectively) and CYP11B1 (1.9±0.3, 3.2±0.8, 4.8±3.2, respectively). The expression of CYP11B2 mRNA was significantly elevated in both the ATII-R APA (13.4±4.9) and ATII-U APA (10.8±1.1) subjects compared with the controls (0.5±0.3). Although the AT1R mRNA levels did not differ between ATII-R APA and ATII-U APA, the AT2R mRNA levels were significantly higher in the ATII-R APA (8.6±3.5) than in ATII-U APA group (1.4±0.3) ($p<0.05$; Fig. 3).

The AT1R or AT2R mRNA levels were parallel with the protein expression levels of each receptor by Western blot analysis of the adrenals of normal controls (data not shown).

Discussion

Our results showed that the upright posture increased p-aldo with a relatively high frequency (50%). Nomura *et al*. (*11*) also reported a high frequency of ATII-R APA (79%). However, Gordon (*12*) and Biglieri *et al*. (*13*) both reported low frequencies of ATII-R APA. In recent years, the number of patients with IHA who show ATII-dependent aldosterone production has been increasing (*2*). Thus the finding of an increase in p-aldo after standing should be applied cautiously to the differentiation of APA and IHA.

Adrenal mineral corticoid production is mainly regulated by the systemic RAS. In the adrenal glands, it has been shown that renin and angiotensinogen and angiotensin-converting enzyme are expressed, and together compose a local RAS. In experiments carried out in rats with bilateral nephrectomies and resulting low PRA, the animals were found to have increased p-aldo and marked up-regulation of CYP11B2 in the adrenals (*14*). The varied concentration and expression of renin in both normal and pathological adrenal tissues were reported by Racz *et al*. (*15*). They detected mRNA in one of two APAs, and in two of the three normal adrenal glands by Northern blot. Klemm *et al*. (*16*) reported that renin mRNA expression was increased in ATII-R APA compared with ATII-U APA and found no mutation of the renin gene in ATII-R APA. In our study, we compared the expression of renin and angiotensinogen mRNA and found no significant difference in the expression of renin or angiotensinogen mRNA among ATII-R APA, ATII-U APA, and normal adrenal tissues.

Cook *et al*. (*17*) demonstrated a predominance of AT1R and increased binding sites of ATII in APA with a defect in ATII-stimulated aldosterone secretion. Elevated expression of AT1R mRNA in APA has also been reported (*18*). We previously analyzed the AT1R gene in aldosteronoma tissues and found no mutation (*4*). These results suggest that post-transcriptional abnormalities in AT1R may exist in APA.

In the present study, the levels of AT2R mRNA were significantly higher in subjects with ATII-R APA than in those with ATII-U APA. Although both AT2R and AT1R were highly expressed in the human zona glomerulosa cells, the pathophysiological roles of AT2R are controversial. In humans and rodents, ATII acting *via* AT1R activates phospholipase C, which causes a rise in 1,4,5-inositol triphosphate and diacylglycerol, resulting in activation of protein kinase C and Ca^{2+} channels (*19*). AT2R acts mainly through Gi and tyrosine phosphatases to exert predominantly inhibitory actions in cellular responses mediated by the AT1R (*20*). However, it has also been shown that the AT2R acts in concordance with the AT1R in collagen synthesis in cultured vascular smooth muscle cells (*21*), in proliferative effects in the mesenteric artery (*22*), in smooth muscle cell growth and extracellular matrix expression in the aorta (*23*), and in inflammation and apoptosis in IgA nephropathy (*24*). Tanabe *et al*. (*25*) reported that not only AT1R mRNA but also AT2R mRNA was highly expressed in APA, and that treatment with an AT2R agonist, CGP-42112, increased aldosterone production in APA. These authors also reported that treating rats with an AT1R blocker, candesartan, for a long period increased p-aldo (this is known as the aldosterone breakthrough phenomenon), and that this increase in p-aldo could be blocked by treatment with an AT2R blocker, PD123319 (*26*). In rodents, type 1b angiotensin receptor (AT1bR) mRNA is predominantly expressed in the adrenal zona glomerulosa. Chen *et al*. (*27*) reported that p-aldo was increased by a low salt diet in both AT1bR null mice and control mice. Taken together, these results suggest that adrenal AT2R may be able to take over the role of AT1R in pathological states. However, the signaling pathways that influence the aldosterone synthesis activated by the AT2R are uncertain. Recently, many genes that are regulated in a ligand-dependent or independent manner by AT2R have been reported using a microarray method (*28*). Our results suggest that AT2R may have stimulatory effects on aldosterone secretion in ATII-R APA *via* the regulation of the responsiveness to ATII. Further studies will be needed to clarify the pathophysiological roles of the AT2R including the signaling pathways.

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