Diverse responses of autoantibodies to the angiotensin II type 1 receptor in primary aldosteronism

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Abstract

Primary aldosteronism (PA) is a common form of endocrine hypertension mainly caused by a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH). Autoantibodies that activate the angiotensin II type 1 receptor (AT1R-Abs) have been reported in patients with disorders associated with hypertension. Our objective was to assess AT1R-Ab levels in patients with PA (APA, n=40; BAH, n=40) relative to patients with primary hypertension (n=40), preeclampsia (n=23) and normotensive individuals (n=25). AT1R-Abs in whole sera were measured using 2 different ELISAs which gave contrasting results. A functional cell-based assay was used to quantify activation of the angiotensin II type 1 receptor (AT1R) using whole sera or affinity-purified antibodies in the absence or presence of losartan (a specific AT1R antagonist). Serum samples from all groups displayed different levels of AT1R activation with different responses to losartan. Patients with BAH displayed higher losartan-independent affinity-isolated agonistic AT1R-Ab levels compared with patients with APA ($P<0.01$) and with normotensive individuals ($P<0.0001$). In patients with APA, BAH and PH combined, higher aldosterone-to-renin ratios and lower plasma renin concentrations were associated with higher compared with lower agonistic AT1R-Abs levels. In patients with PA, higher AT1R-Ab activity was associated with an increased likelihood of a diagnosis of BAH compared with APA and with the presence of adrenal hyperplasia detected by computed tomography. Taken together these data suggest that agonistic AT1R-Abs may have a functional role in a subgroup of patients with primary aldosteronism.

Introduction

Primary aldosteronism (PA) is a form of endocrine hypertension caused by the overproduction of aldosterone from one or both adrenal glands (unilateral or bilateral PA, respectively). Unilateral PA is predominantly caused by an aldosterone-producing adenoma (APA) and bilateral forms by bilateral adrenocortical hyperplasia (BAH).[1] APA and BAH mainly arise sporadically but uncommon familial forms have been described (familial hyperaldosteronism types I-IV).[2,3] Substantial progress has been made in understanding the pathophysiology of familial PA and sporadic APAs with the identification of germline mutations causing 4 familial forms of hyperaldosteronism[4-9] and somatic mutations which drive aldosterone excess in 50-80% of APAs.[2,10-12] These advances, however, have not been replicated in understanding the pathogenesis of sporadic BAH. The bilateral nature of the disease led to the proposal of circulating factors, which could trigger bilateral chronic stimulation of the adrenal *zona glomerulosa*.

Graves disease is an established example of an autoimmune disease caused by agonistic autoantibodies which activate the thyroid stimulating hormone receptor (TSHR) resulting in thyroid hormone production and thyroid cell proliferation.[13-15] In addition to agonistic antibodies, antagonistic and neutral autoantibodies to the TSHR have been described which either block TSH activity or have no apparent effect.[15] Autoimmune responses to other G protein coupled receptors have been reported in several studies implicating a role for autoantibody activation of the angiotensin II type 1 receptor (AT1R), the α_1-adrenergic and β_1-adrenergic receptors in several cardiovascular disorders.[16-25] Furthermore, multiple studies have reported the detection of autoantibodies to the angiotensin II type 1 receptor (AT1R-Abs) in patients with preeclampsia.[20,26] AT1R-Abs which recognize the AFHYESQ peptide (position 165-171) in the second extracellular loop of the AT1R have been implicated as autoantibody-mediated drivers of AT1R agonism.

Specifically, ELISAs employing an immobilized synthetic AFHYESQ peptide are often used to assay AT1R-Ab levels.[20,27] Using either ELISA or functional assays, AT1R-Abs have also been reported in patients with PA in whom AT1R-Ab levels are variously reported as higher in patients with APA than with BAH, higher in BAH compared with APA or similar levels in both subtypes of PA.[28-30] These studies have either used ELISA-based assays, which do not provide information on the agonistic effect of AT1R-Abs, or have included only small cohorts of patients with PA.

Our objective was to establish if functionally active AT1R-Abs are present in a large cohort of 80 patients with PA (40 patients with APA, 40 with BAH) in comparison with primary hypertension (PH, n=40), preeclampsia (PE, n=23) and normotensive individuals (NT, n=25) using 3 assays: 2 different ELISA-based assays both using immobilized full-length AT1R and a highly sensitive cell-based AT1R activation functional assay.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Methods

Patient samples

For quantification of AT1R-Abs and AT1R activating activity, serum samples from 80 patients with PA (40 with APA and 40 with BAH), 40 with primary hypertension (PH), 23 women with preeclampsia (PE) and 25 normotensive blood donors (NT) were used. PA was diagnosed in accordance with the Endocrine Society guideline.[31] Patients were screened for PA using the plasma aldosterone-to-direct renin concentration ratio and diagnosis was confirmed by the intravenous saline load test according to local criteria.[32] All patients with confirmed PA underwent computed tomography (CT) scanning and adrenal venous sampling. The cut-off selectivity index to determine success of catheterization was ≥ 2 and for the lateralization index to diagnose unilateral PA ≥ 4.[32] PH was diagnosed in accordance with the ESH/cardiology guidelines[33] after ruling out PA, pheochromocytoma and Cushing syndrome. PE and Graves disease were diagnosed as described previously.[34,35] Blood sampling for patients with PA and PH was performed at screening for secondary hypertension. Whenever possible, patients were under no treatment or before the beginning of an anti-hypertensive therapy. When necessary blood pressure was controlled using the calcium channel blocker verapamil or the α-blocker doxazosin, alone or in combination, in accordance with the ES guideline.[31] Blood samples from patients with Graves disease were withdrawn at the first medical visit and from patients with PE in the third trimester. All participants gave written informed consent and the protocol was approved by the local ethics committee.

AT1R autoantibody measurements

All AT1R-Abs were measured using 3 different assays. Two commercially available ELISA kits were used to quantify autoantibodies against the recombinant human full-length AT1R (ELISA-Creative Diagnostics and ELISA-CellTrend).[36, 37] The third assay was a cell based AT1R activation assay (Invitrogen Gene BLAzer beta-lactamase reporter system) to measure agonistic AT1R activity in total serum and in affinity-isolated IgG fractions after pre-incubation for 1 hour with vehicle or 100 μM losartan. Immunoglobulins (IgGs) were affinity isolated on protein A/G agarose from 1 mL patient serum and 1/10 of the affinity-isolated IgGs was used in the functional assay. The isolation of IgGs on protein A/G agarose and their depletion from serum samples was confirmed by Western blotting using a horseradish peroxidase conjugated goat anti-human antibody (Millipore, 1:50000 dilution) (**Figure S1**).

The cell based AT1R activation assay employed AT1R-*bla* U2OS cells which stably express the AT1R linked at the C-terminus to the Gal4-VP16 transcription factor via a TEV (Tobacco Etch Virus) protease cleavage site (E-X-X-Y-X-Q-G/S) (Invitrogen). The U2OS cells also stably express TEV protease-tagged-β-arrestin/TEV and a β-lactamase reporter gene with Gal4-responsive upstream activator sequences. Following AT1R activation, the TEV-protease-β-arrestin is recruited to the AT1R receptor C-terminus and cleaves the TEV cleavage sequence releasing GAL4-VP16 which activates expression of the β-lactamase reporter gene. A Förster resonance energy transfer (FRET) substrate comprising coumarin and fluorescein fluorophores was used to measure reporter gene activity (ThermoFisher, LiveBLAzer-FRET B/G substrate). In the absence of β-lactamase reporter gene expression, the FRET substrate is intact, coumarin excitation transfers fluorescence resonance energy to fluorescein resulting in emission of green fluorescence. When the substrate is cleaved, energy transfer is disrupted and a blue fluorescence signal is emitted from coumarin excitation. Reporter activities, corresponding to AT1R activation, are given as response ratios which are the ratio of coumarin to fluorescein fluorescence signals (ratio of cleaved to uncleaved substrate) normalized for negative control wells (mock-treated cells).

TSHR activation assay

Activity of affinity-isolated IgGs from serum of Graves disease patients was measured using a TSHR agonistic cell-based assay to determine if autoantibody functional activity was maintained following the IgG isolation procedure. The assay uses TSHR ACTOne cells, a HEK-293 CNG (human embryonic kidney-293 cyclic nucleotide gated) cell line with overexpression of recombinant human TSHR (MyBiosource). The modified CNG channel opens in response to elevated intracellular cAMP levels and the resultant ion influx and membrane depolarization is measured with a fluorescent membrane potential dye. The assay measures intracellular cAMP levels as a response ratio between TSHR ACTOne cells compared with the parental control cell line (HEK-293 CNG cells).

Adrenal morphology

CT imaging was used to classify absence or presence of adrenal hyperplasia in adrenals with an abnormal morphology. The absence of hyperplasia group included adrenals with an adenoma but without hyperplasia, the presence of hyperplasia group included adrenals with hyperplasia alone or hyperplasia and an adenoma. Hyperplasia was defined as mean limb width ≥ 5 mm.[38] Patients with no adrenal abnormality visible on CT images were excluded from the morphologic analysis.

Statistical analyses

Data were analyzed with the Kolmogorov-Smirnov and Shapiro-Wilk tests to determine distributions. Group differences were calculated for normally distributed data using the ANOVA and post-hoc Bonferroni tests. Non-normally distributed data were analyzed using the Kruskal-Wallis test. Accordingly, data are expressed as mean ± SD or median (25^{th} to 95^{th} percentile). Categorical variables are presented as absolute numbers and percentages and differences were analyzed with a Chi-square test. Adjusted logistic regression analyses were performed to assess associations of AT1R-Abs and the diagnosis of BAH. IBM SPSS Statistics version 22.0 was used for all analyses.

Results

Clinical parameters of patients with primary aldosteronism versus primary hypertension

Groups of patients with APA and BAH had the same age as patients with PH and a similar gender distribution with no significant differences in the proportion of males and females between APA, BAH and PH groups (47.5-57.9%). There were no significant between-group differences in systolic or diastolic blood pressure at baseline or in body mass index in patients with APA, BAH and PH (**Table 1**). As expected, patients with APA or BAH had higher plasma aldosterone concentrations (PAC) and lower direct plasma renin concentrations (DRC) at baseline relative to the PH group (PAC: APA group, 569 [283-1071]; BAH, 416 [311-583]; PH 225 [128-394] pmol/L and DRC: APA group, 4.3 [2.0-11.2]; BAH, 3.4 [2.0-7.3]; PH, 18.2 [8.9-45.1] mU/L). Likewise, patients with APA had lower serum potassium concentrations compared with patients with BAH and PH (APA group, 2.9 [2.6-3.2]; BAH, 3.3 [3.0-3.7]; PH 3.9 [3.6-4.2] mmol/L) (**Table 1**).

ELISA quantification of AT_1R-Abs in different groups

Autoantibodies recognizing epitopes on the full-length human recombinant AT1R in serum from patients with APA, BAH, PH, PE and NT were measured using 2 different ELISAs. Using one approach (ELISA-Creative Diagnostics), patients with PE displayed significantly higher AT1R-Ab levels compared with all other groups (P<0.0001 for all comparisons). The titer of AT1R-Abs was highly similar in the APA and BAH groups (APA group, [0.06-0.21]; BAH, 0.12 [0.06-0.26] ng/mL) with no differences observed compared with either the PH or NT groups (PH group, 0.15 [0.10-0.25]; NT, 0.11 [0.01-0.19] ng/mL) (**Figure, panel A; Table S1**). We also used a second ELISA (ELISA-CellTrend) based on AT1R-Ab binding to the full-length AT1R in its native conformation.[36, 37] Patients with APA and BAH displayed highly similar levels of AT1R-Abs (APA group, 14.2 [10.4-22.0]; BAH, 14.1 [10.1-19.7] U/mL) which were not significantly different from the PH or NT groups (PH group, 13.5 [10.7-18.7]; NT, 11.4 [10.6-20.8] ng/mL) (**Figure, panel B, Table S1**). However, AT1R-Ab levels were significantly lower in patients with PE (8.7 [6.9-11.6] ng/mL) compared with all other groups (P<0.05 for all comparisons).

Quantification of AT1R agonistic activity in serum samples from different groups

We tested if serum from the different subgroups of patients and individuals could activate the AT1R in a cell based functional assay. Treatment of cells with angiotensin II (0-500 pM) demonstrated a dose-dependent effect on AT1R activation which was ablated by pre-incubation of the cells for 1 h with the AT1R antagonist losartan (100 μM). The assay measured a specific AT1R functional response to 50 pM angiotensin II which was significantly higher than a corresponding incubation in the presence of losartan (P<0.05) (**Figure S2**). Higher AT1R agonistic activity was measured in serum samples from all groups (P<0.001 for absence *versus* presence of losartan for each group). There were no between-group differences for AT1R agonist activity in the absence of losartan. However, in the presence of losartan there were overall differences in the measured functional activation of the AT1R (P<0.001) with the BAH group showing higher activity compared with the APA (P=0.001), PE (P<0.0001) and NT groups (P<0.0001). The PH group also displayed higher levels of functional AT1R-Abs relative to the NT (P<0.0001) and the PE groups (P=0.001) **(Figure, panel C, Table S1)**.

Affinity isolation of IgG fractions from different groups of serum samples

To determine if the losartan-independent AT1R activating activity in serum samples was due to IgGs or to other circulating factors, such as angiotensin II, IgGs were affinity-isolated from all serum samples on protein A/G-agarose to assess AT1R agonist activity in the cell based AT1R functional assay (**Figure S1, Figure S2**). We first tested if the IgG affinity-isolation procedure produced functionally active autoantibodies. For this, IgGs were isolated from the serum of patients with Graves disease (N=9) and measured TSHR activation using a cell based functional assay. Using IgG fractions isolated from patients with Graves disease, comparison of TSHR activation in the ACT-ONE cell line (with stable overexpression of the human TSHR) with the parental cell line (without expression of recombinant human TSHR) demonstrated that 6 of the 9 IgG fractions displayed TSHR agonistic activity (**Figure S3**). The remaining 3 IgG fractions exhibited no significant TSHR activation indicating neutral or blocking activity to the TSHR (**Figure S3**). Overall, the approach used for the affinity isolation of autoantibodies from patients with Graves disease maintained TSHR agonist functional activity thereby validating the method used for the isolation of IgG fractions.

Quantification of AT1R agonistic activity in affinity-isolated IgG fractions from different groups

There were group differences in the cell-based assay response (overall difference *P*<0.001) using affinity-isolated IgGs. The BAH, PH and PE groups displayed higher levels of AT1R activating autoantibodies compared with the NT group (*P*<0.0001, *P*=0.007 and *P*<0.0001, respectively) and the BAH group had higher functional AT1R-Ab levels than the APA group (*P*=0.01). The agonistic AT1R-Ab levels were not abolished in the presence of losartan and significant group differences were observed (**Table S1**). Higher losartan-independent AT1R functional activity was measured with IgGs isolated from patients with BAH, PH and PE compared with the NT group (*P*<0.0001, *P*=0.006 and *P*=0.016, respectively) and in the BAH *versus* APA groups (*P*=0.01) (**Figure, panel D, Table S1**). Comparison of AT1R activation in the cell assay with the functional response obtained with angiotensin II in the dose-response assay indicated that the median AT1R activation achieved with affinity-isolated antibodies from patients with BAH in the presence or absence of losartan was equivalent to 50 to 100 pM angiotensin II (**Figure S2, Table S1**).

Clinical parameters of patients according to functional AT1R-Ab levels

Affinity-purified agonistic AT1R-Ab levels were categorized into higher and lower AT1R-Ab levels according to the median AT1R-Ab activity in the cell-based assay for patients with APA, BAH and

PH combined. In this cohort, in the absence of losartan, patients with BAH had higher AT1R-Ab levels (BAH represented 41.2% of 68 patients of the combined cohort with higher AT1R-Ab levels compared with 23.1% of 52 patients with lower AT1R-Ab levels, *P*=0.037) (**Table 2**). Patients with APA had lower AT1R-Ab levels (APA represented 23.5% of 68 patients of the combined cohort with higher AT1R-Ab levels compared with 46.2% of 52 patients with lower AT1R-Ab levels, *P*=0.009) (**Table 2**). Although functional AT1R-Ab levels were similar in the BAH *versus* PH groups (**Figure, panel D; Table S1**), patients with PH with lower *versus* higher AT1R-Ab levels were similarly distributed in the combined cohort (APA + BAH + PH). The PH group with lower AT1R-Ab levels comprised 30.7% of 52 patients of the combined cohort compared with 35.3% of 68 patients with higher levels (*P*=0.603) (**Table 2**).

In the APA, BAH and PH combined cohort, higher levels of agonistic AT1R-Abs were also associated with a higher aldosterone-to-renin ratio (ARR_DRC) and a lower direct renin concentration (DRC) in the absence of losartan (DRC: 5.7 mU/mL [2.2-27.0] *versus* 11.7 mU/mL [5.7-31.8], *P*=0.011; ARR_DRC: 47 [13-139] *versus* 23 [10-55], *P*=0.029, for higher *versus* lower AT1R-Ab levels, respectively) and these differences were maintained in the presence of losartan (**Table 2**).

Patients with PA with higher agonistic AT1R-Ab levels, in the absence of losartan, had an increased likelihood of a diagnosis of BAH *versus* APA after adjustment for confounding effects of age, systolic BP, PAC or DRC (**Table 3**). Higher losartan-independent agonistic AT1R-Ab levels were not associated with a diagnosis of BAH compared with APA after correction for systolic BP and PAC. There was no association of higher AT1R-Ab levels with a diagnosis of BAH compared with PH in either the absence of presence of losartan (**Table 3**).

Adrenal morphology according to functional AT1R-Ab levels

Adrenal abnormalities were absent on CT images in 3 patients diagnosed with APA and in 17 patients diagnosed with BAH, and these cases were excluded from the morphologic analysis. Higher affinity-purified AT1R-Ab levels in the absence of losartan were associated with the presence of adrenal hyperplasia when AT1R-Ab levels were treated as either a continuous variable (AT1R activating activity response ratio, 0.3 [0.26-0.39] *versus* 0.26 [0.23-0.29] in the presence and absence of hyperplasia, respectively, *P*=0.011) or categorized as higher or lower according to the median AT1R-Ab level (76.0 % of 25 patients with adrenal hyperplasia had higher AT1R-Ab levels compared with 37.1% of 35 patients without adrenal hyperplasia, *P*=0.003) (**Table 4**). In the presence of losartan, AT1R-Ab activities were similar in the presence *versus* absence of hyperplasia groups (**Table 4**).

The distribution of individual patients with PA (APA and BAH) with adrenal hyperplasia according to AT1R-Ab activating activity is shown in **Figure, panel D.** In patients with PA, 83.3% of 12 and 69.2% of 13 patients of patients classified with adrenal hyperplasia in the APA and BAH groups, respectively, had AT1R-Ab levels above or equal to the median value for their group in the absence of losartan (**Figure, panel D**).

Discussion

Autoantibodies that potentially elicit a functional response by binding to G protein-coupled receptors have been described in several cardiovascular disorders.[25] Many studies have reported AT1R-Ab binding to an epitope in the second extracellular loop (AFHYESQ) of the AT1R in different groups of patients.[20] The best characterized is AT1R-Abs in PE where a functional role has been implicated using cardiomyocyte contraction assays in which assay response was ablated either by the AT1R antagonist losartan or with the AFHYESQ peptide.[20,39] The prevalence of AT1R-Abs in PE varies widely with reports employing an ELISA ranging from 48% of 58 patients[40] to 100% of 25 patients.[20] However, targeting the AFHYESQ peptide in ELISA assays has limitations because binding to linear immobilized peptides may not correlate with AT1R agonism and binding to conformational epitopes cannot be assessed.[41] A commercially available ELISA (ELISA-CellTrend), routinely used in solid organ transplantation, has been developed based on autoantibody binding to the full-length AT1R in the native conformation.[37] Using this conformation sensitive assay, we demonstrated highly contrasting low AT1R-Ab levels compared with a different ELISA method which appears to greatly overestimate the level of AT1R-Abs in patients with preeclampsia.

The pathophysiology of sporadic BAH is poorly understood. Advances in knowledge are hampered by the highly limited availability of tissue samples for molecular studies because patients with BAH are not usually surgically-treated. Despite this, recent studies have suggested a role for adrenocortical hyperplasia in patients with bilateral but asymmetrical inappropriate aldosterone production[42] or a role for small clusters of cells located beneath the adrenal capsule with high aldosterone synthase expression (called aldosterone-producing cell clusters) in surgically-treated patients diagnosed with bilateral PA.[43] Thus, BAH may not be a distinct entity but a disorder comprising clinical and biochemical variability arising from morphological heterogeneity representing the variable response of the adrenal cortex to circulating, environmental and genetic factors.

A role for autoantibodies that trigger bilateral chronic stimulation of the adrenal *zona glomerulosa* via activation of the AT1R has been proposed[44] but a pathogenic role for AT1R-Abs in PA remains unclear because of conflicting reports that used different methods for assessment of antibody levels.[28-30] One study found a 2-fold increase of AT1R-Abs against the AFHYESQ peptide in an ELISA in patients with APA (n=26) compared with patients with BAH (n=20) and proposed the use of this assay as a potential diagnostic tool to differentiate the two different types of PA.[28] Using a similar ELISA-based AFHYESQ assay no difference in AT1R-Ab levels were observed in 44 patients with PA (15 with APA, 29 with BAH) compared with 18 normotensive individuals (n=18) and no difference in AT1R-Ab levels between the patients with APA and BAH.[30] However, measuring antibody binding to the linear AFHYESQ peptide in ELISA assays, as used in many studies, does not necessarily correlate with AT1R agonism.

To address the agonistic activity of AT1R-Abs in PA, Kem et al[29] reported increased AT1R-Ab levels in patients with PA (n=13) compared with control subjects (n=20) using cell-based assays to measure a functional response in AT1R-transfected cells and reported the contractile effects of the isolated IgGs in perfused rat cremaster arterioles. In contrast to other reports, an increased prevalence of AT1R-Abs in patients with BAH relative to patients with APA was reported.[29] However, the number of patients with PA assessed for AT1R-Ab levels was small, the stimulating activity of low potency and the affinity-isolated antibodies did not elicit a dose-dependent functional effect.[29]

The diverse observations for the prevalence and potential role of AT1R-Abs and the limited understanding of the pathogenesis of bilateral PA highlight the need for studies to measure autoantibodies using robust functional assays in large and well characterized cohorts of patients with PA. Herein, we assessed AT1R-Ab levels in a cohort of 80 patients with PA diagnosed in accordance with rigorous criteria and with subtype diagnosis (APA *versus* BAH) defined by adrenal venous sampling. Following this approach, ELISA-based measurements using the immobilized full-length AT1R gave contrasting results for AT1R-Ab levels in patients with PE and did not reveal statistical differences between patients with BAH or APA compared with PH or NT. We hence also used a cell-based AT1R functional assay which exploits specific activation of the β-lactamase reporter gene upon ligand binding to the AT1R. With this assay, similar levels of AT1R activation were measured in whole serum from all groups. However, between-group differences were shown using affinity-isolated IgGs which demonstrated significantly higher levels of agonistic AT1R-Abs in patients with BAH compared with APA and in patients with BAH, PH and PE relative to the NT group both in the presence and absence of losartan.

These activities implicate the existence of an alternative epitope structurally remote from losartan binding sites. AT1R is increasingly recognized as a multi-ligand binding surface and epitopes discovered in solid organ transplant patients are not identical with those in patients with PE[37]. Some reports suggest that, in addition to classical G protein-mediated signaling, "biased" AT1R signaling mediated by β-arrestin[45,46] may play a role in aldosterone production and have pathological implications for the progression to heart failure after myocardial infarction.[47,48] Because losartan antagonizes G protein signaling but is ineffective in ablating β-arrestin-mediated signaling,[47,48] the losartan-independent activity we report presumably comprises "biased" AT1R signaling.

We also demonstrate that higher agonistic AT1R-Ab levels are associated with clinical parameters characteristic of autonomous aldosterone production in PA such as higher aldosterone-to-renin ratios and lower plasma renin levels. The degree of functional activity of AT1R-Abs in this study appears low but is potentially pathologically relevant because the median AT1R-Ab agonistic activity in patients with BAH corresponds to greater than that achieved with 50 pM angiotensin II, a concentration similar to plasma angiotensin II concentrations reported in patients with chronic kidney disease and considerably higher than in healthy individuals.[49]

A potential pathogenic role of agonistic AT1R-Abs in PA is suggested by the association of higher active AT1R-Ab levels - in the absence but not in the presence of losartan - with an increased likelihood of a diagnosis of BAH compared with APA and with an increased incidence of adrenal hyperplasia. Adrenals harboring an APA also often display focal or diffuse cortical hyperplasia adjacent to the adenoma.[42,50] It is notable that within the group of patients with APA, those with evidence of hyperplasia at CT scanning tend to display higher levels of AT1R-Ab agonistic activity compared with patients with APA without hyperplasia. The imaging data should however be treated with caution considering the potential for incorrect classification of an adenoma *versus* hyperplasia.

Taken together the present data indicate that AT1R-Abs may play a role in patients with BAH which could feasibly exacerbate the effects of additional pathophysiological factors such as aldosterone-producing cell clusters which have been reported as larger, more numerous and with a higher prevalence of aldosterone-driver mutations than normal adrenals.[43] Notwithstanding the observations reported herein, the possibility that AT1R-Abs are a marker of hypertension rather than having a pathogenic role cannot be excluded.

In conclusion, some patients with disorders related to hypertension have activating autoantibodies to the AT1R. Some AT1R-Abs function via a mechanism diverse from the classical G protein-mediated AT1R signaling and implicate a role for losartan-independent “biased” AT1R signaling. Overall, the present study suggests a role for agonistic autoantibodies to the AT1R in a subgroup of patients with PA, comprising those patients with adrenal hyperplasia,

Perspectives

A role for AT1R-Abs has been implicated in several cardiovascular disorders but evidence for a direct function in disease pathophysiology is lacking. *In vivo* experiments in mice subjected to infusion of AT1R-Abs from patients with PA could clarify the impact of AT1R-Abs on aldosterone production. A longitudinal analysis is planned to measure the response of AT1R-Ab levels to adrenal surgery or mineralocorticoid receptor antagonism in patients with PA with long term follow up. Epitope mapping using synthetic peptides to competitively abolish autoantibody-mediated AT1R activation will aid the identification of AT1R-Ab binding sites and establish any role for autoantibodies in “biased” signaling.

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Conflicts of Interest Disclosure

None

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Novelty and Significance

What is New?

- AT1R-Ab levels were measured in groups of patients with hypertension compared with normotensive individuals
- Higher agonistic AT1R-Abs levels were present in bilateral primary aldosteronism, primary hypertension and preeclampsia groups compared with normotensive individuals
- Patients with bilateral *versus* unilateral primary aldosteronism had higher levels of agonistic AT1R-Abs

What is relevant?

- AT1R-Abs measured by ELISA did not correlate with functional activation of the AT1R
- Patients with higher AT1R-Ab activity levels have an increased likelihood of a diagnosis of bilateral than unilateral primary aldosteronism
- Higher levels of agonistic AT1R-Abs were associated with higher aldosterone-to-renin ratios and lower plasma renin concentrations
- Patients with primary aldosteronism with adrenal hyperplasia displayed higher agonistic AT1R-Abs levels

Summary

Agonistic autoantibodies to the AT1R are present in patients with disorders related to hypertension and may contribute to autonomous aldosterone production and adrenal hyperplasia in a subgroup of patients with primary aldosteronism

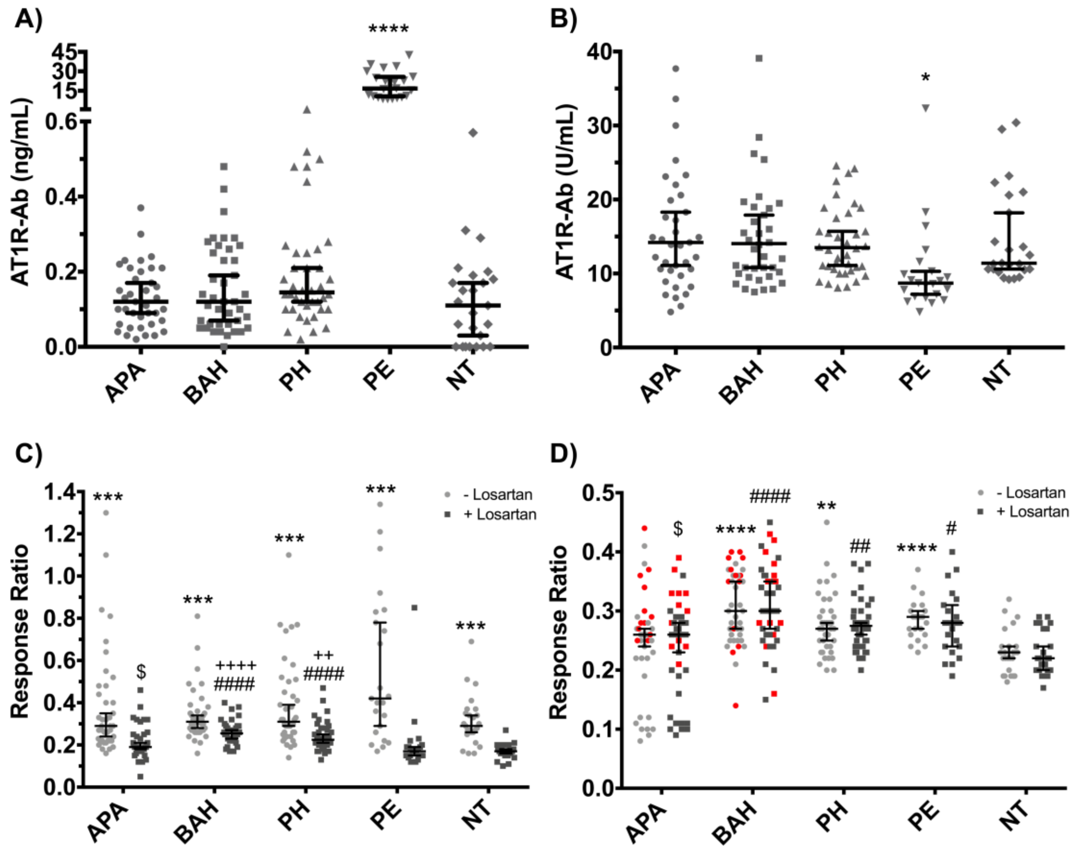

Figure. Measurement of AT1R autoantibodies and AT1R activating response in patients with primary aldosteronism, primary hypertension, preeclampsia and in normotensive individuals

Scatter dot plots showing quantification of AT1R-Ab in total serum of patients with PA (APA and BAH), PH, PE and normotensive individuals by measurements using ELISA-Creative Diagnostics (**Panel A**) or ELISA-CellTrend (**Panel B**). A cell-based AT1R activation assay was used to measure AT1R-Ab agonist activity in total serum (**Panel C**) or in agarose-A/G affinity isolated IgG fractions (**Panel D**) in the absence (**light grey points**) or presence (**dark grey points**) of 100 µm losartan as indicated. **Panel D** also highlights the agonistic AT1R-Ab levels in patients with adrenal hyperplasia at CT imaging (**red points**). The response ratio represents AT1R-activation of β-lactamase activity measured as coumarin to fluorescein fluorescence (cleaved to uncleaved substrate ratio) normalized for negative controls. Horizontal lines within boxes indicate the median, and the lower and upper horizonal lines indicate the 95% CI. *P* values were calculated using the Kruskal-Wallis test and indicate **** difference (P<0.0001) from NT (**Panel A**); * difference (P<0.05) from NT (**Panel B**); *** difference (P<0.001) absence *versus* presence of losartan for each subgroup; $^{\$}$ difference (P<0.01) from BAH; $^{\#\#\#\#}$ difference (P<0.0001) from NT (presence of losartan); $^{++++}$ difference (P<0.0001) from PE (presence of losartan); $^{++}$ difference (P<0.01) from PE (presence of losartan); (**Panel C**); ** difference (P<0.01) from NT (absence of losartan), **** difference (P<0.0001) from NT (absence of losartan); $^{\$}$ difference (P<0.01) (presence of losartan); $^{\#\#\#\#}$ difference (P<0.0001) from NT (presence of losartan); $^{\#\#}$ difference (P<0.01) from NT (presence of losartan); $^{\#}$ difference (P<0.05) from NT (presence of losartan); (**Panel D**). Numbers of patient samples in each subgroup were APA, *N*=40; BAH, *N*=40; PH, *N*=40; PE, *N*=23; NT, *N*=25. APA, aldosterone-producing adenoma; AT1R-Ab, angiotensin II type 1 receptor autoantibodies; BAH, bilateral adrenal hyperplasia; PH, primary hypertension; PE, preeclampsia; NT, normotensive individuals.

Clinical parameter	APA (*N*=40)	BAH (*N*=40)	PH (*N*=40)	Overall *P*-value	*Pairwise comparisons*		
					APA *vs* BAH	APA *vs* PH	BAH *vs* PH
Age (years)	52 ± 10.2	52 ± 9.7	52 ± 19.9	0.964	N.A.	N.A.	N.A.
Sex (ref. male)	21 (52.5%)	19 (47.5%)	16 (42.1%)	0.656	N.A.	N.A.	N.A.
BMI (Kg/m^2)	27.3 ± 4.1	26.2 ± 5.0	27.4 ± 6.0	0.500	N.A.	N.A.	N.A.
Systolic BP (mmHg)	151 ± 21.5	151 ± 23.8	156 ± 17.2	0.461	N.A.	N.A.	N.A.
Diastolic BP (mmHg)	93 ± 11.0	95 ± 13.6	91 ± 14.6	0.469	N.A.	N.A.	N.A.
PAC (pmol/L)	569 [283-1071]	416 [311-583]	225 [128-394]	< 0.001	0.742	< 0.001	0.002
DRC (mU/L)	4.3 [2.0-11.2]	3.4 [2.0-7.3]	18.2 [8.9-45.1]	< 0.001	0.831	< 0.001	< 0.001
ARR_DRC	108 [36-306]	114 [71-162]	16 [6-26]	< 0.001	1.000	< 0.001	< 0.001
Lowest serum K^+ (mmol/L)	2.9 [2.6-3.2]	3.3 [3.0-3.7]	3.9 [3.6-4.2]	< 0.001	0.001	< 0.001	< 0.001

Table 1. Clinical parameters of patients with primary aldosteronism and primary hypertension

Clinical data of patients with PA (APA or BAH) and PH are presented as average values ± SD, absolute numbers with proportions in parenthesis (%) or as medians with lower and upper quartiles in parentheses. *P* values designate the presence of group differences by the ANOVA and Bonferroni post-hoc tests (age, BMI, systolic and diastolic BP), Kruskal–Wallis test (PAC, DRC, ARR_DRC and potassium), or Chi square test (sex). Numbers of patient samples in each subgroup are indicated. APA, aldosterone-producing adenoma; ARR_DRC, aldosterone-to-renin ratio using direct renin measurements; BAH, bilateral adrenal hyperplasia; BMI, body mass index; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension.

Clinical parameter	AT1R-Ab level minus losartan		*P*-value	AT1R-Ab level plus losartan		*P*-value
	< median	≥ median		< median	≥ median	
Diagnosis: APA	24 (46.2)	16 (23.5)	0.009	23 (40.4)	17 (27.0)	0.120
BAH	12 (23.1)	28 (41.2)	0.037	14 (24.6)	26 (41.3)	0.053
PH	16 (30.7)	24 (35.3)	0.603	20 (35.1)	20 (31.7)	0.699
Age (years)	54 ± 14.8	55 ± 16.6	0.749	54 ± 15.5	55 ± 16.2	0.851
Sex (ref. male)	30 (57.7)	39 (57.4)	0.970	28 (49.1)	41 (65.1)	0.077
BMI (Kg/m^2)	28.2 ± 4.7	27.5 ± 5.0	0.431	27.2 ± 4.2	28.4 ± 5.3	0.177
Systolic BP (mmHg)	151 ± 23.9	147 ± 19.2	0.376	148 ± 25.1	149 ± 17.4	0.709
Diastolic BP (mmHg)	92 ± 15.0	86 ± 12.5	0.018	89 ± 16.9	89 ± 10.7	0.854
PAC (pmol/L)	235 [150-553]	300 [167-556]	0.499	236 [130-550]	286 [186-569]	0.338
DRC (mU/L)	11.7 [5.7-31.8]	5.7 [2.2-27.0]	0.011	11.9 [5.3-39.7]	5.6 [2.3-16.3]	0.003
ARR_DRC	23 [10-55]	47 [13-139]	0.029	19 [7-60]	49 [16-137]	0.003
Lowest serum K^+ (mmol/L)	3.2 [2.9-3.9]	3.4 [3.2-3.9]	0.333	3.3 [2.9-3.9]	3.4 [3.2-3.9]	0.084

Table 2. Clinical parameters of patients with primary aldosteronism and primary hypertension according to functional AT1R-Ab levels

Clinical parameters of the combined cohort of patients with APA, BAH and PH were analyzed according to AT1R-Ab levels (affinity-purified autoantibody activity measured with the cell-based assay) categorized according to the median value of the combined cohort (median values, 0.27 and 0.28 in the absence and presence of losartan respectively). Data are presented as average values ± SD, absolute numbers with proportions in parenthesis (%) or as medians with lower and upper quartiles in parentheses. *P* values designate the presence of group differences by the ANOVA and Bonferroni post-hoc tests (age, BMI, systolic and diastolic BP), Kruskal–Wallis test (PAC, DRC, ARR_DRC and potassium), or Chi square test (sex, diagnosis). Numbers of patient samples in each subgroup are indicated. APA, aldosterone-producing adenoma; ARR_DRC, aldosterone-to-renin ratio using direct renin measurements; BAH, bilateral adrenal hyperplasia; BMI, body mass index; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension.

Clinical parameter	BAH *vs.* APA		BAH *vs.* PH	
	OR (CI 95%)	*P*-value	OR (CI 95%)	*P*-value
Agonistic AT1R-Ab level - losartan				
AT1R-Abs (ref. ≥ median)	3.425 (1.342-8.696)	0.010	1.515 (0.589-3.891)	0.388
Age (years)	0.976 (0.941-1.012)	0.186	1.025 (0.997-1.053)	0.078
AT1R-Abs (ref. ≥ median)	3.663 (1.420-9.434)	0.007	1.495 (0.587-8.817)	0.339
Systolic BP (mmHg)	1.019 (0.005-1.044)	0.116	0.993 (0.972-1.015)	0.532
AT1R-Abs (ref. ≥ median)	3.521 (1.361-9.091)	0.009	1.887 (0.688-5.319)	0.231
PAC (pmol/L)	1.001 (1.000-1.003)	0.072	1.003 (1.001-1.005)	0.003
AT1R-Abs (ref. ≥ median)	3.546 (1.395-9.009)	0.008	1.603 (0.630-4.065)	0.322
DRC (mU/L)	0.996 (0.989-1.004)	0.298	0.996 (0.990-1.002)	0.221
Agonistic AT1R-Ab level + losartan				
AT1R-Abs (ref. ≥ median)	2.571 (1.027-6.452)	0.044	1.980 (0.786-5.000)	0.147
Age (years)	0.973 (0.938-1.009)	0.135	1.026 (0.999-1.055)	0.062
AT1R-Abs (ref. ≥ median)	2.358 (0.943-5.882)	0.066	1.832 (0.745-4.505)	0.187
Systolic BP (mmHg)	1.015 (0.992-1.039)	0.211	0.993 (0.971-1.014)	0.497
AT1R-Abs (ref. ≥ median)	2.381 (0.947-5.988)	0.065	2.278 (0.838-6.211)	0.107
PAC (pmol/L)	1.001 (1.000-1.002)	0.086	1.003 (1.001-1.005)	0.003
AT1R activation (ref. ≥ median)	2.500 (1.007-6.211)	0.048	1.698 (0.678-4.255)	0.258
DRC (mU/L)	0.966 (0.989-1.004)	0.323	0.997 (0.990-1.003)	0.314

Table 3. Association of agonistic affinity-purified AT1R-Ab levels and diagnosis of BAH

Logistic regression analyses were performed to determine the potential association of agonistic autoantibody levels with a diagnosis of BAH with adjustment for confounding effects of a single clinical variable per level (age, systolic BP, PAC or DRC) in the absence and presence of losartan. Autoantibody levels were categorized according to the median affinity-purified AT1R-Ab level in the cell-based assay as shown. Data are presented as odds ratios (OR) with 95% confidence intervals (CI). An OR > 1 indicates an increased likelihood for a diagnosis of BAH in the presence of agonistic AT1R-Ab activity ≥ median value independent of the tested confounding variable (age, systolic BP, PAC, DRC). APA, aldosterone-producing adenoma; AT1R, angiotensin II type 1 receptor; BAH, bilateral adrenal hyperplasia; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension; ref, reference.

Clinical parameter	Hyperplasia		P-value
	Absence (n=35)	Presence (n=25)	
Diagnosis			
APA	25 (71.4)	12 (48.0)	0.066
BAH	10 (28.6)	13 (52.0)	
Agonistic AT1R-Ab level - losartan			
AT1R-Abs (response ratio)	0.26 [0.23-0.29]	0.30 [0.26-0.39]	0.011
AT1R-Abs (ref. ≥ median)	13 (37.1)	19 (76.0)	0.003
Agonistic AT1R-Ab level + losartan			
AT1R-Abs (response ratio)	0.27 [0.20-0.30]	0.30 [0.24-0.36]	0.149
AT1R-Abs (ref. ≥ median)	16 (45.7)	15 (60.0)	0.205

Table 4. Functional AT1R autoantibody levels stratified by adrenal morphology

Adrenal morphology of patients with PA was determined from CT results to classify absence or presence of hyperplasia in adrenals with morphologic abnormalities. Numbers of patient samples in each subgroup are indicated. Affinity-purified agonistic AT1R-Ab levels, measured with the cell-based assay, were treated as continuous variables and presented as medians with lower and upper quartiles in parenthesis or categorized as higher and lower agonistic AT1R-Ab levels according to the median value for patients with APA and BAH combined and presented as absolute numbers with proportions in parenthesis. *P* values designate the presence of group differences by the Kruskal–Wallis test (AT1R-Ab levels), or Chi square test (diagnosis, AT1R-Ab levels after categorization).