

Novel Retro-Inverso Peptide Inhibitor Reverses Angiotensin Receptor Autoantibody–Induced Hypertension in the Rabbit

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Abstract—Activating autoantibodies to the angiotensin II type 1 receptor (AT1R) have been implicated in hypertensive disorders. We investigated whether AT1R antibodies produced in immunized rabbits will activate AT1R and contribute to hypertension by a direct contractile effect on the vasculature and whether they can be blocked by a novel decoy peptide. A multiple antigenic peptide containing the AT1R epitope AFHYESQ, which is the receptor-binding epitope of AT1R-activating autoantibodies, was used to immunize 6 rabbits. AT1R antibody activity was analyzed in AT1R-transfected cells, and their contractile effects were assayed using isolated perfused rat cremaster resistance arterioles. A retro-inverso D-amino acid epitope-mimetic peptide was tested for AT1R antibody inhibition in vitro and in vivo. All immunized animals produced high AT1R antibody titers and developed elevated blood pressure. No changes in measured blood chemistry values were observed after immunization. Rabbit anti-AT1R sera induced significant AT1R activation in transfected cells and vasoconstriction in the arteriole assay, both of which were blocked by losartan and the retro-inverso D-amino acid peptide. A single intravenous bolus injection of the retro-inverso D-amino acid peptide (1 mg/kg) into immunized rabbits dropped the mean arterial pressure from 122±11 to 82±6 mm Hg. Rabbit anti-AT1R sera partially suppressed angiotensin II–induced contraction of isolated rat cremaster arterioles, and the pressor response to angiotensin II infusion was attenuated in immunized animals. In conclusion, AT1R-activating autoantibodies and the retro-inverso D-amino acid peptide, respectively, have important etiologic and therapeutic implications in hypertensive subjects who harbor these autoantibodies. (*Hypertension*. 2015;65:793-799. DOI: 10.1161/HYPERTENSIONAHA.114.05037.)

Key Words: hypertension ■ rabbit ■ receptor, angiotensin, type 1 ■ retro-inverso peptide
■ vasoconstriction

Hypertension is a major risk factor for cardiovascular and renal diseases with high morbidity and mortality. It affects ≈50 million people in the United States and imposes a tremendous health and economic burden on society.¹ Despite the availability of numerous antihypertensive medications, the control of blood pressure remains inadequate in many cases. The causation of essential hypertension, the most common form of hypertension, is complex and incompletely understood. Multiple mechanisms have been proposed to contribute to its pathogenesis. Recent evidence from both clinical and basic studies suggests that hypertension may have an autoimmune basis.^{2,3} Autoantibodies to the angiotensin II (Ang II) type 1 receptor (AT1R) have been described in patients with preeclampsia,⁴ malignant and refractory hypertension,^{5,6} renal allograft rejection,⁷ and in subjects with primary aldosteronism.^{8,9} These autoantibodies demonstrated agonistic activity in vitro, and their titers correlated with disease severity.¹⁰

More importantly, transfer of AT1R-activating autoantibodies (AT1R-AAbs) from patients with preeclampsia to nonpregnant and pregnant mice, respectively, produced hypertension and a preeclampsia-like phenotype, both of which were prevented by the AT1R blocker losartan.¹¹ Agonistic autoantibodies to the α 1-adrenergic receptor (α 1AR) have also been documented in patients with essential and refractory hypertension.^{12–14} In animal models, immunization with α 1AR-derived receptor peptide induced cardiac remodeling and diastolic dysfunction associated with α 1AR-activating antibodies developed in the rats.^{15,16} However, these α 1AR-immunized animals failed to develop hypertension.

The heptapeptide sequence AFHYESQ from the second extracellular loop of AT1R has been identified as the functional epitope of AT1R-AAbs from patients with preeclampsia.⁴ We have used a multiple antigenic peptide containing this epitope sequence to immunize the rabbit and demonstrated for

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the first time an AT1R-AAb-induced hypertensive phenotype in immunized animals. This study used this animal model of autoimmune hypertension to investigate the therapeutic potential of a newly designed retro-inverso D-amino acid (RID) decoy peptide that specifically targets AT1R-AAbs. RID peptides, in which D-amino acids are substituted for L-amino acids in a reversed sequence, assume a side-chain topology similar to that of their parent peptides but with inverted amide peptide bonds. They mimic the structure and antigenicity of the parent L-peptide but are resistant to protease degradation.¹⁷ Here, we demonstrate that the RID peptide can effectively block the effects of AT1R-AAbs both in vitro and in vivo.

Methods

This study protocol was approved by the Institutional Animal Care and Use Committee of the Oklahoma City Veterans Affairs Medical Center and Oklahoma University Health Sciences Center and conformed to international standards for animal safety and comfort.

Experimental Procedures

Six New Zealand white rabbits (2.5–3 kg), fed on standard rabbit chow, were immunized with 1 mg of a multiple antigenic peptide containing the AT1R epitope sequence AFHYESQ (GenScript, Piscataway, NJ) in 0.5 mL of complete Freund's adjuvant. The animals were boosted with the same peptide plus incomplete Freund's adjuvant (1 mg/0.5 mL) at 2 and 4 weeks. At 6 weeks, the rabbits were treated with an intravenous bolus injection (1 mg/kg) of an epitope-mimicking RID peptide (d-QSEYHFA; GenScript). Under anesthesia (ketamine/xylazine, 35 mg/5 mg/kg), the rabbit central ear artery was cannulated and the catheter was connected to a pressure transducer (Edwards Lifesciences, Irvine, CA). Arterial blood pressure was measured at preimmune and postimmune (6 weeks after immunization) before and 90 minutes after RID peptide injection. To determine the acute effect of Ang II on blood pressure before and after

immunization, increasing doses of Ang II (10, 100, and 500 ng/kg) were injected intravenously at 5-minute intervals using an infusion pump, and the blood pressure response at each dose was recorded. Each rabbit served as its own control. Preimmune and postimmune sera were obtained from all animals for ELISA and activity assays of the expected antibodies generated during immunization.

Peptide Stability to Proteolysis

The stability of the peptides in serum was assessed by incubation of the peptides (500 µg) in human serum (500 µL) at 37°C. After a specific time, a sample (200 µL) was added to a centrifugal filter of molecular weight cutoff 10K and centrifuged for 15 minutes at 12000 rpm. A sample of filtrate (100 µL) was injected into a high-pressure liquid chromatography system (Beckman, Pasadena, CA) for analysis.

Blood Chemistry Analysis

Arterial blood samples were analyzed with a portable analyzer (E3+ cartridges, VetScan i-STAT Handheld Analyzer; Abaxis Veterinary Diagnostics, Union City, CA) for immediate measurement of sodium, potassium, chloride, hematocrit, and hemoglobin.

ELISA

Antibodies produced in the sera were detected by ELISA. Briefly, microtiter plates were coated with the AT1R multiple antigenic peptide at 10 µg/mL. To determine the antibody titer, sera were diluted to 1:10000 and thereafter diluted 2 fold. Goat antirabbit IgG conjugated with alkaline phosphatase and its substrate para-nitrophenyl-phosphate 104 were used to detect antibody binding. Titers were determined as the highest dilution with an optical density value of 0.10 at 60 minutes.

Cell-Based AT1R Activation Assay

Agonist-like autoantibody activity was measured in AT1R-transfected Chinese hamster ovary cells using the PathHunter eXpress β-arrestin

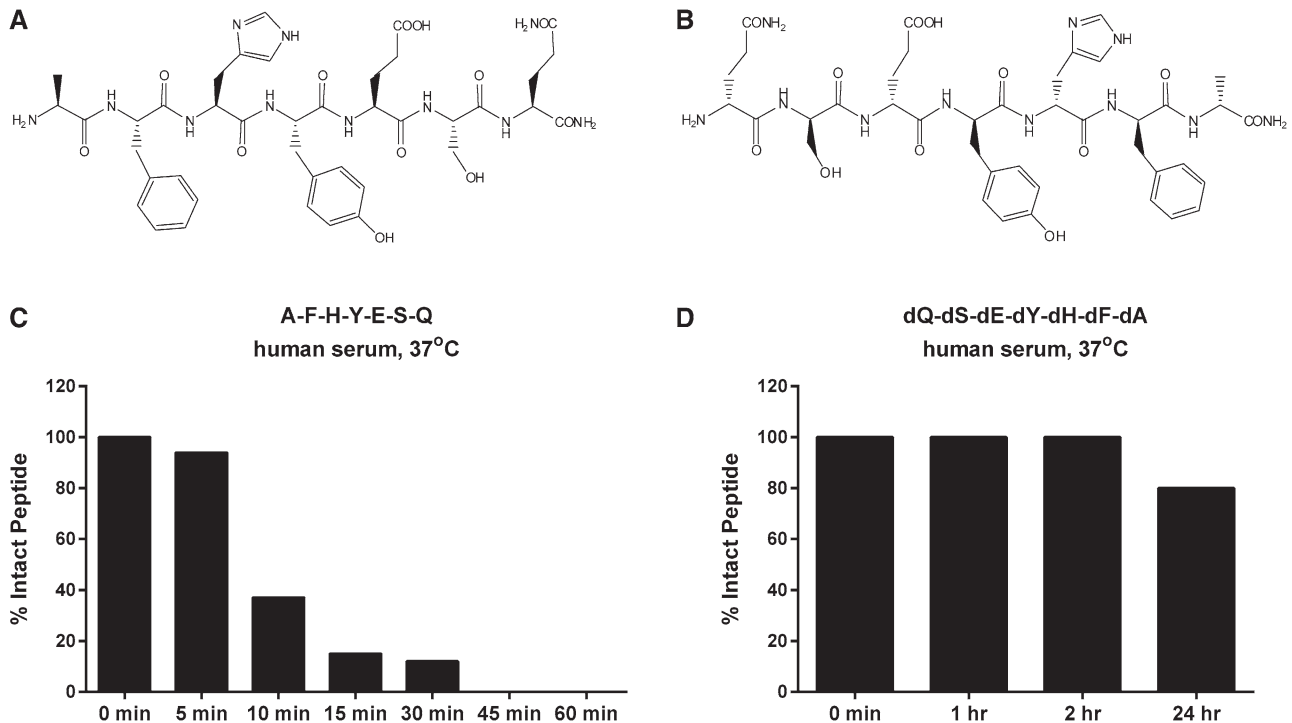


Figure 1. Chemical structure and serum stability of the angiotensin II type 1 receptor epitope peptide (A and C) and epitope-mimetic retro-inverso D-amino acid (RID) peptide (B and D). There was a rapid proteolysis of the L-amino acid peptide AFHYESQ in human serum in vitro with complete degradation by 45 minutes, whereas the RID peptide d-QSEYHFA remained intact at 2 hours and 80% was still intact at 24 hours.

GPCR assay kit (DiscoverX, Fremont, CA) as previously described.⁹ Briefly, 10 000 AT1R-Chinese hamster ovary cells were dispensed into each well of a 96-well culture plate and incubated for 48 hours. Assay buffer containing rabbit sera (1:20–1:500) in the presence and absence of AT1R blocker losartan (10 μ M) was then added and incubated for 90 minutes. Preincubation of rabbit anti-AT1R sera with an excess of the RID peptide was also tested for neutralization studies. Ang II (100 nmol/L) was used as a positive control. All samples were tested in triplicate. After sample treatment, PathHunter detection reagents were added and the luminescence signal was quantitated to determine β -arrestin recruitment levels. The β -arrestin values are expressed as percentage of buffer baseline to normalize the individual data.

Contractility Assay

Antibody pressor activity was assayed using an isolated rat cremaster arteriole assay as previously described.⁹ Briefly, arteriolar segments were microdissected from the cremaster muscle of anesthetized rats and cannulated with glass micropipettes. After equilibration and development of steady-state myogenic tone, the arterioles were perfused with rabbit sera (1:10–1:500). The AT1R blocker losartan (10 μ M) was then added to the perfusate, and the effect on vessel diameter was recorded. The effect of rabbit anti-AT1R sera preincubated with the RID peptide was also tested. A dosage response curve for Ang II (10^{-10} to 10^{-5} mol/L) in the absence and presence of rabbit anti-AT1R sera (1:100) was constructed to examine the effect of AT1R-AAbs on Ang II-induced contractile response. Measurements of vessel diameter were made using a video edge detector. Data are reported as percentage of baseline diameter to normalize each response.

Statistical Analysis

Data are presented as mean \pm SEM. Comparison between 2 groups was performed by paired or unpaired Student *t* test as appropriate. One-way ANOVA followed by Newman–Keuls post hoc test was used for multiple group comparisons. Statistical significance was set at $P<0.05$.

Results

Development of a Proteolytically Resistant Peptidomimetic

We designed a RID peptide based on the known AT1R epitope sequence AFHYESQ. This enantiomer peptide, made of all D-amino acids in a reversed sequence (d-QSEYHFA), maintains a side-chain topology similar to that of the original L-amino acid peptide, thus mimicking the molecular structure of the peptide AFHYESQ (Figure 1A and 1B). The stability of the RID peptide in human serum was analyzed by high-pressure liquid chromatography to quantify the intact peptide remaining in solution. In comparison with the L-amino acid peptide AFHYESQ, which was rapidly degraded with complete loss of integrity by 45 minutes, the RID peptide remained 100% intact at 2 hours and 80% intact at 24 hours (Figure 1C and 1D).

Inhibition of AT1R-AAb-Induced Hypertension by RID Peptide

The mean arterial pressure (MAP) was measured at preimmune baseline and 6 weeks after initial immunization. Each rabbit served as its own control. The MAP was significantly elevated after immunization with the AT1R epitope peptide AFHYESQ (preimmune: 73 ± 5 versus postimmune: 122 ± 11 mmHg; $P<0.01$; Figure 2A). The postimmune heart rate was also significantly increased compared with baseline (pre-immune: 137 ± 7 versus postimmune: 165 ± 8 bpm; $P<0.01$;

Figure 2B). A single bolus injection of the RID peptide at 6 weeks effectively suppressed the elevated MAP (postimmune: 122 ± 11 versus post-RID: 82 ± 6 mmHg; $P<0.05$; Figure 2A) and heart rate (postimmune: 165 ± 8 versus post-RID: 140 ± 8 bpm; $P<0.05$; Figure 2B) to their baseline levels. A similar study at 12 weeks demonstrated a sustained suppression of blood pressure elevation in the absence of any further interventions (data not shown).

Blood levels of sodium, potassium, chloride, hematocrit, and hemoglobin in all rabbits were analyzed, and no significant differences in any of the measured parameters were detected between the preimmune and postimmune states (Table).

Inhibition of AT1R-AAb Activity by RID Peptide In Vitro

All 6 rabbits developed high antibody titers to AT1R ranging from 1:640 000 to 1:1.28 million after peptide immunization. The ability of rabbit sera to stimulate AT1R activation was tested in transfected Chinese hamster ovary cells in vitro. The postimmune sera demonstrated a dose–response effect on AT1R activation with significant activity at dilutions ranging from 1:20 to 1:200 (Figure 3A). The postimmune sera (1:100) induced a significant increase in AT1R activity compared with the preimmune sera ($250\pm 17\%$ versus $131\pm 9\%$; $P<0.01$), which was effectively blocked by the AT1R blocker losartan (10 μ M) and by preincubation with the RID peptide (from $250\pm 17\%$ to $158\pm 15\%$ and $150\pm 11\%$, respectively; $P<0.01$;

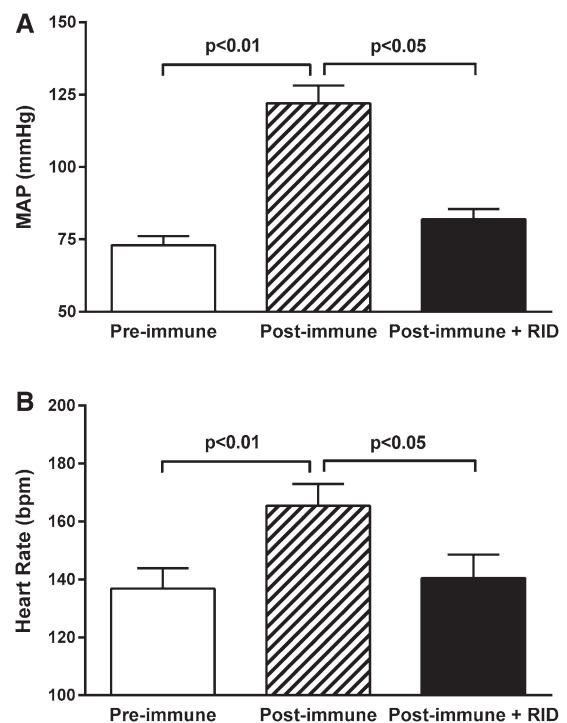


Figure 2. Effect of the retro-inverso D-amino acid (RID) peptide on angiotensin II type 1 receptor (AT1R)-activating autoantibody-induced elevation of blood pressure (A) and heart rate (B) in the rabbit. Rabbits ($n=6$) were immunized with the AT1R epitope peptide and boosted at 2 and 4 weeks. At 6 weeks, there was a significant elevation in mean arterial pressure (MAP) and heart rate compared with the preimmune state. Injection of the immunized animals with the RID peptide (1 mg/kg) at 6 weeks reversed the elevated MAP and heart rate to their baseline levels.

Table. Comparison of Blood Chemistry Values From the 6 Rabbits Before and After Immunization

Analyte	Preimmune	Postimmune	P Value
Sodium, mEq/L	141±2	144±3	0.10
Potassium, mEq/L	3.7±0.1	3.5±0.3	0.13
Chloride, mEq/L	100±3	103±4	0.12
Hematocrit, %	34±2	35±3	0.49
Hemoglobin, g/dL	11.7±0.6	11.9±1.1	0.46

Figure 3B). No significant AT1R activity was found with the preimmune sera compared with buffer baseline.

The vasoconstrictive response to rabbit anti-AT1R sera was examined using a rat cremaster arteriolar assay. There was a significant dose effect on vasoconstriction for the postimmune sera with maximal activity at 1:10 dilution (Figure 4A). Significant contractile activity was observed at dilutions ranging from 1:10 to 1:125. The postimmune sera (1:100)-induced arteriole contractility was largely suppressed by losartan (10 μ M) and by preincubation with the RID peptide (from 87±3% to 97±3% and 95±3%, respectively; P <0.01; Figure 4B). No significant contractile activity was observed in the preimmune sera.

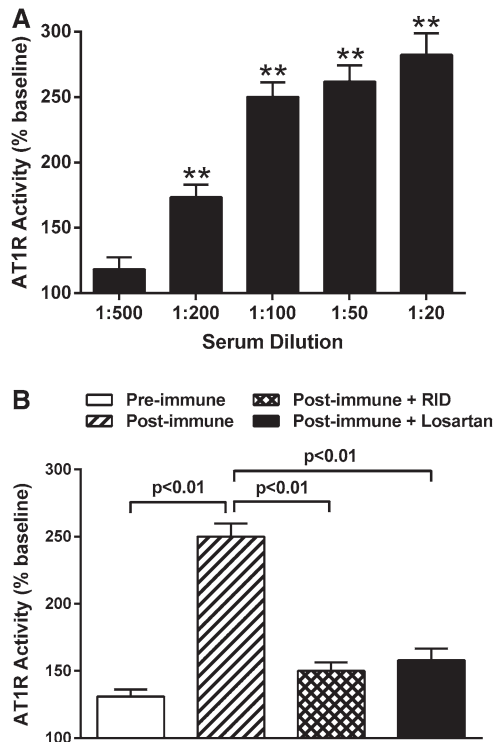


Figure 3. A, Dose effect of rabbit postimmune antiangiotensin II type 1 receptor (AT1R) sera on AT1R activation in transfected Chinese hamster ovary (CHO) cells. Dilutions as low as 1:200 produced a significant increase in AT1R activity compared with the buffer alone. $^{**}P$ <0.01 vs buffer baseline, $n=3$. **B**, Effect of the retro-inverso α -amino acid (RID) peptide and losartan on AT1R-activating autoantibody-induced AT1R activation in transfected CHO cells. Compared with the preimmune sera, the postimmune sera (1:100) from the 6 rabbits led to significant activation of AT1R, which was suppressed by the RID peptide and AT1R blocker losartan (10 μ M). Data are expressed as percentage of buffer baseline.

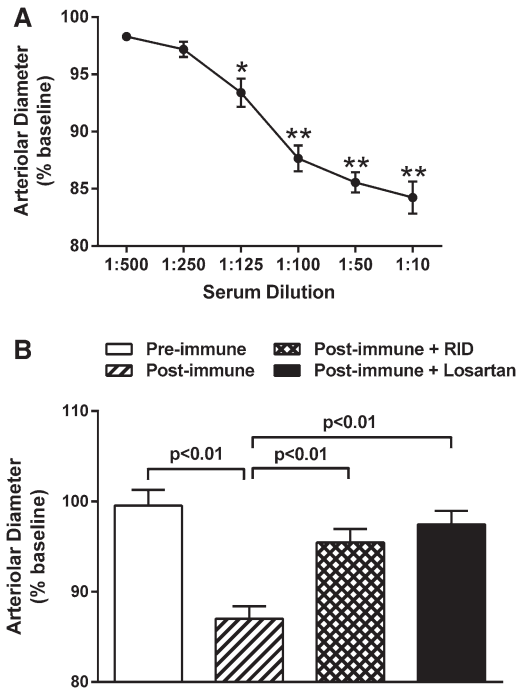


Figure 4. A, Dose effect of rabbit postimmune antiangiotensin II type 1 receptor (AT1R) sera on vasoconstriction in the rat cremaster arteriole assay. There was significant contractile activity in the postimmune sera at dilutions from 1:10 to 1:125. $^{*}P$ <0.05 and $^{**}P$ <0.01 vs buffer baseline, $n=4$. **B**, Effect of the retro-inverso α -amino acid (RID) peptide and losartan on AT1R-activating autoantibody-induced contraction of isolated rat cremaster arterioles. Compared with the preimmune sera, the postimmune sera (1:100) from the 6 rabbits induced significant vasoconstriction, which was effectively blocked by the RID peptide and losartan (10 μ M). Data are expressed as percentage of buffer baseline.

Allosteric Effect of AT1R-AAbs on Ang II-Mediated Pressor Response

To determine whether AT1R-AAbs would alter the hemodynamic responses produced by Ang II injections, we infused increasing doses (10, 100, and 500 ng/kg) of Ang II into rabbits before and after immunization and compared their MAP values. Ang II infusion caused transient, dose-dependent increases in MAP in both preimmune and postimmune states; however, the magnitude of the pressor response in the postimmune state was significantly less than that in the preimmune state (Figure 5A). At doses of 100 and 500 ng/kg, Ang II caused an increase in MAP of 17.8±2.5 and 23.3±2.7 mm Hg, respectively, in the postimmune state compared with 31.6±2.8 and 51.5±3.8 mm Hg, respectively, in the preimmune state (P <0.01). The effect of rabbit anti-AT1R sera (1:100) on Ang II-induced vasoconstriction was also examined using the cremaster arteriole assay. As shown in Figure 5B, the contractility curve for Ang II (10^{-10} to 10^{-5} mol/L) was shifted significantly to the right in the presence of anti-AT1R sera compared with control, demonstrating a similar negative allosteric effect of AT1R-AAbs as observed in vivo.

Discussion

The renin-angiotensin system is integrally involved in cardiovascular and renal homeostasis; therefore, the

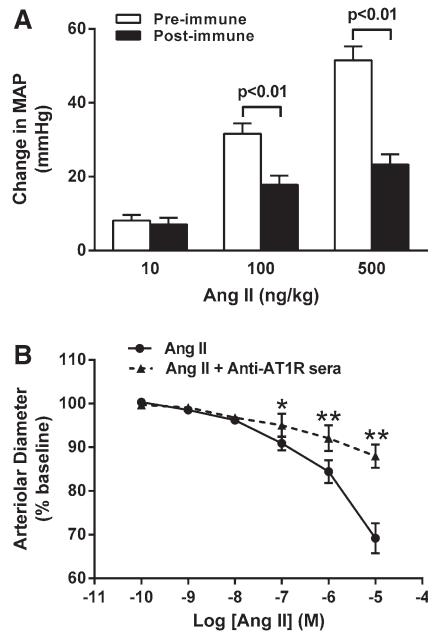


Figure 5. Effect of antiangiotensin II (Ang II) type 1 receptor (AT1R)-activating autoantibodies on Ang II-induced pressor response in the rabbit (A) and in isolated rat cremaster arterioles (B). Infusion of the 6 rabbits with increasing doses of Ang II (10, 100, and 500 ng/kg) before immunization caused significantly greater increases in mean arterial pressure (MAP) than those observed at 6 weeks post immunization. In the cremaster vasoconstriction assay, rabbit anti-AT1R sera (1:100) shifted the Ang II dose-response curve to the right, demonstrating an allosteric antagonistic effect of AT1R-activating autoantibodies. * $P < 0.05$ and ** $P < 0.01$, $n = 3$.

presence of circulating Ang II-like autoantibodies would be expected to play a role in altered regulation of blood pressure and cardiac function. AT1R autoantibodies have been implicated in several types of hypertensive disorders. However, no models have been established to provide direct evidence linking hypertension to these autoantibodies as opposed to the indirect or observed associations described clinically. Wenzel et al¹⁸ used the heptapeptide AFHYESQ to immunize the rabbit, which induced the production of AT1R-AAbs. The short-term passive transfer of the rabbit AT1R-AAbs alone in their pregnant rats did not alter the rat blood pressure. However, coinfusion of relatively low dosages of Ang II along with the AT1R-AAbs did raise the blood pressure in their rat model of preeclampsia. The effect of AT1R-AAbs alone on blood pressure in their immunized rabbits was not reported. When we immunized our rabbits with the same peptide, all animals produced AT1R-AAbs that functioned similarly to those observed in humans; and all animals demonstrated significantly elevated blood pressure. In contrast to Wenzel et al,¹⁸ these AAbs caused the animals to be resistant to Ang II infusion. These data suggest that there are inherent differences between the non-pregnant and pregnant responsiveness to both Ang II and AT1R-AAbs. Our model, therefore, provides a valuable tool for in vivo studies of pathophysiology and treatment of autoantibody-mediated hypertension without the need for coexistent infusion of Ang II.

Investigation of therapeutic strategies that target AAbs in cardiovascular disease has primarily focused on cardiomyopathy associated with autoantibodies to the β 1-adrenergic receptor (β 1AR). These include elimination of cardiomyopathic autoantibodies by immunoadsorption^{19,20} and use of specific peptides or oligonucleotides as autoantibody scavengers.²¹⁻²³ Orthosteric receptor antagonists are questionable because they also block the normal ligand and thereby impair the normal physiological responses. Small peptide inhibitors as therapeutic agents have several advantages compared with other small molecules, including high specificity and low levels of toxicity and immunogenicity.²⁴ The use of a modified β 1AR second extracellular loop cyclic peptide to neutralize β 1AR-AAbs has been advocated and is under limited trial in Europe.²⁵ In this study, we tested a proteolytically stable RID peptide designed to target AT1R-AAbs. This peptide demonstrated effective antibody-blocking effect both in vitro and in vivo. It inhibited AT1R-AAb-stimulated AT1R activation in cultured cells, blocked AT1R-AAb-induced contraction of isolated resistance arterioles, and reversed the elevated blood pressure for ≥ 3 months in AT1R-immunized animals. RID peptides offer the advantage of increased biological half-life and bioactivity compared with the natural L-peptides. This approach has been used effectively for the development of peptide inhibitors as a potential novel treatment for Alzheimer disease²⁶ and lupus.²⁷ Compared with the cyclic peptide approach that requires complex synthesis and uses an entirely different means of protection of the peptide structure, this construct is relatively simple and can be easily adapted to targets of other autoantibodies and administered orally.

We have previously reported that AT1R-AAbs have a variable effect on different tissues. AT1R-AAbs harvested from our rabbits by themselves demonstrated a contractile effect on an isolated arteriole in vitro, which is compatible with their hypertensive effects in vivo. AT1R-AAbs harvested from patients with primary aldosteronism were capable of stimulating aldosterone production in vitro and presumably had a similar effect in vivo.⁹ The high sodium intake in our rabbits led to suppressed plasma renin activity values in the basal state. The small number and variability of aldosterone values obtained in the postimmune state prevented us from drawing a significant conclusion about stimulation of aldosterone production. The small drop in serum potassium that was observed was not significant. We are planning more detailed studies on the metabolic consequences of AT1R immunization.

Allosteric autoantibodies may express variable modulatory effects on the action of the orthosteric natural ligand, in some circumstances acting as enhancers and in other circumstances as attenuators.²⁸ We have previously reported that β 1/2AR-AAbs from patients with postural tachycardia syndrome facilitated β AR agonist isoproterenol-stimulated β 1/2AR activation in cultured cells in vitro, whereas α 1AR-AAbs from these patients partially blocked α 1AR agonist phenylephrine-induced vasoconstriction in the isolated rat cremaster arteriole assay.²⁹ The differing allosteric effects of these autoantibodies may contribute to the pathophysiology

of postural tachycardia syndrome. Using the same arteriole assay, we have now demonstrated that AT1R-AAbs from immunized rabbits exerted a similar antagonistic effect on Ang II–induced contractile response. Consistent with this finding, the pressor response to Ang II infusion was attenuated in immunized rabbits compared with the preimmune baseline. It is not clear at this point whether these allosteric effects will be consistent for a given autoantibody–receptor complex or whether a variety of autoantibodies directed toward nearby structures will provide a kaleidoscope of effects when studied in detail.

Perspectives

We have developed a rabbit model of AT1R-AAb–mediated hypertension. This model will be useful in studying auto-immune hypertensive pathophysiology, as well as therapeutic interventions. We used a reconstructed peptide that is capable of binding to the antibodies but with conferred protection against proteolysis. This peptide therefore serves as a decoy and prevents the autoantibody from binding to the cell membrane receptor and keeping it from activation. This would lead to clearance of the decoy peptide–autoantibody complex and potentially lead to the body developing tolerance to this epitope and suppression of autoantibody production. Pharmacological blockade of pathological autoantibodies with novel peptide inhibitors will set the stage for developing a new strategy for the prophylaxis or treatment for patients who harbor these autoantibodies and presumably would leave the targeted receptor free for normal physiological function.

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Disclosures

D.C. Kem as an inventor has submitted US Patent no. 7348305, Application no. PCT/US14/28362, about the retro-inverso D-amino acid peptide used in this article.

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Novelty and Significance

What Is New?

- Immunization of rabbits with a peptide from the angiotensin II type 1 receptor (AT1R) produced AT1R-activating autoantibody-mediated hypertension, which was blocked by a proteolytically stable decoy peptide that specifically targets the AT1R autoantibodies.

What Is Relevant?

- AT1R-activating autoantibodies have been reported in patients with hypertensive disorders, including preeclampsia, malignant and refractory hypertension, and primary aldosteronism.

Summary

This study supports a pathophysiological role of AT1R-activating autoantibodies in hypertension. Pharmacological blockade of pathological autoantibodies with peptide inhibitors may open a new avenue for developing novel therapeutic approaches for patients who harbor these autoantibodies.

Novel Retro-Inverso Peptide Inhibitor Reverses Angiotensin Receptor Autoantibody-Induced Hypertension in the Rabbit

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