Effectiveness and Safety of a Therapeutic Vaccine Against Angiotensin II Receptor Type 1 in Hypertensive Animals

Xiao Chen,* Zhihua Qiu,* Shijun Yang,* Dan Ding,* Fen Chen, Yanzhao Zhou, Min Wang, Jibin Lin, Xian Yu, Zihua Zhou, Yuhua Liao

Abstract—Primary hypertension is a chronic disease with high morbidity, and the rate of controlled blood pressure is far from satisfactory, worldwide. Vaccination provides a promising approach for treatment of hypertension and improvement in compliance. Here, the ATRQβ-001 vaccine, a peptide (ATR-001) derived from human angiotensin II (Ang II) receptor type 1 conjugated with Qβ bacteriophage virus-like particles, was developed and evaluated in animal models of hypertension. The ATRQβ-001 vaccine significantly decreased the blood pressure of Ang II–induced hypertensive mice up to 35 mm Hg (143±4 versus 178±6 mm Hg; P=0.005) and that of spontaneously hypertensive rats up to 19 mm Hg (173±2 versus 192±3 mm Hg; P=0.003) and prevented remodeling of vulnerable hypertensive target organs. No obvious feedback activation of circulating or local renin-angiotensin system was observed. Additionally, no significant immune-mediated damage was detected in vaccinated hypertensive and nonhypertensive animals. The half-life of the anti–ATR-001 antibody was 14.4 days, surpassing that of existing chemical drugs. In vitro, the anti–ATR-001 antibody specifically bound to Ang II receptor type 1 and inhibited Ca2+-dependent signal transduction events, including protein kinase C-α translocation, extracellular signal-regulated kinase 1/2 phosphorylation (72% decrease; P=0.013), and elevation of intracellular Ca2+ (68% decrease; P=0.017) induced by Ang II, but without inhibiting Ang II binding to the receptor. In conclusion, the ATRQβ-001 vaccine decreased the blood pressure of Ang II–induced hypertensive mice and spontaneously hypertensive rats effectively through diminishing the pressure response and inhibiting signal transduction initiated by Ang II. Thus, the ATRQβ-001 vaccine may provide a novel and promising method for the treatment of primary hypertension. (Hypertension. 2013;61:408-416.) • Online Data Supplement

Key Words: angiotensin II type 1 receptor ★ hypertension ★ vaccine ★ angiotensin II ★ angiotensin II type 1 receptor autoantibodies

Hypertension has become a leading disease in both developed (333 million) and undeveloped (639 million) countries. The prevalence of hypertension is expected to increase to 29.2% in 2025 (29% of men and 29.5% of women). It is a complex disease caused by multiple factors, 1 of which is the renin-angiotensin system (RAS), which plays a critical role in the development and maintenance of blood pressure. Chemical drugs targeting RAS, including renin inhibitor, angiotensin convert enzyme inhibitor, and angiotensin II (Ang II) receptor blocker (ARB), have exhibited excellent therapeutic effects in clinical practice. Nevertheless, the control rate of blood pressure is still far from being satisfactory worldwide, and the treatment compliance is quite low.

To address the compliance issue and improve therapeutic outcomes for patients, it is necessary to develop novel methods for hypertension treatment. Vaccines can elicit specific antibodies against hypertension related target molecules, providing a possible avenue toward accomplishing this goal. One potential target is the Ang II type 1 receptor (AT1R), a G protein coupling receptor, which has a second extracellular loop (ECL2) that plays an important role in activation of the receptor. The 183Ala and 183His residues in the ECL2 are 2 possible binding sites of Ang II with AT1R. In our previous work, we demonstrated that an epitope from the rat AT1R, designated as ATR12181, can decrease the systolic blood pressure (SBP) of spontaneously hypertensive rats (SHRs) and provide excellent protective effects in target organs. Following our work, another study team emphasized vaccination against AT1R for the prevention of l-NAME–induced nephropathy. Here, a linear epitope ATR-001, derived from the ECL2 of human AT1R including 183Ala and 183His, was designed. To improve the low immunogenicity of ATR-001, it was conjugated with a virus-like particle (VLP) carrier protein, whose remarkable feature is the highly repetitive and ordered surface structure and have been used in the treatment of several chronic diseases.
In this study, the ATR-001 peptide was covalently conjugated with a Qβ bacteriophage VLP as a vaccine (designated ATRQβ-001). We vaccinated Ang II–induced hypertensive Balb/c mice and SHRs to evaluate the antihypertensive effect of ATRQβ-001. Kyoto Wistar rats and normal Balb/c mice were also immunized to assess the safety of the vaccine. Furthermore, the mechanisms of the vaccine in lowering blood pressure of hypertensive animals were investigated.

Materials and Methods
The Methods section is available in the online-only Data Supplement.

Results
ATRQβ-001 Vaccination Effectively Decreases Blood Pressure of Ang II–Induced Hypertensive Mice and SHRs
On day 14, after the second vaccination of mice, when the ATR-001–specific antibody titer was 1:60,000–1:80,000 (Figure 1A), Ang II was infused (1.4 mg/[kg d], SC, n=10), and the SBP was detected periodically. The maximum decline of SBP in mice given the ATRQβ-001 vaccine was 35 mm Hg on day 5 after the Ang II infusion compared with that in the VLP group (143±4 versus 178±6 mm Hg, P=0.005; Figure 1B).

Figure 1. ATRQβ-001 vaccination effectively decreases blood pressure in angiotensin (Ang) II–treated mice and spontaneously hypertensive rats (SHRs). A, Balb/c mice were immunized SC on days 0 and 14, and ATR-001–specific antibody titers were screened on days 14, 20, 27, and 36. B, An osmotic minipump filled with Ang II (1.4 mg/[kg d]) was implanted on day 21 to deliver Ang II for 14 days. ATRQβ-001 vaccination effectively decreased the systolic blood pressure (SBP) in mice infused with Ang II. C, SHRs were immunized on days 0, 14, and 28. ATR-001–specific antibody titers were screened on days 0, 7, 14, 28, 42, 56, 70, 98, and 120. D, The ATRQβ-001 vaccine significantly decreased the SBP of SHRs. E, The antihypertension effect of the ATRQβ-001 vaccine on SHRs was confirmed by telemetry in the morning during a 2-hour period (from 09.00–11.00 hours) on days 42 to 48 (at age 13 weeks). The average SBP of the vaccine SHRs was significantly lower than that of the virus-like particles (VLP) immunization group. All data are expressed as the mean±SE, *P<0.05 and **P<0.01 vs the VLP group.
SHRs (aged 6 weeks, n=9 per group) were used to further evaluate the antihypertensive effects of the ATRQβ-001 vaccine over a long-term observation period. The ATRQβ-001–specific antibody titer in SHRs rose on day 7 after the first vaccination, peaked on day 42 (1:8000–1:16000), and gradually decreased thereafter (Figure 1C). Similar to the variation in antibody responses, SBP levels of SHRs immunized with the ATRQβ-001 vaccine were decreased compared with the VLP immunization group, with a maximum decrease of 19 mm Hg (173±2 versus 192±3 mm Hg, P=0.003; Figure 1D). The maximum blood pressure decrease was similar between the ATRQβ-001 vaccine and valsartan group. However, the blood pressure in the valsartan group decreased much more steadily. To confirm the antihypertensive effect of the ATRQβ-001 vaccine, SBPs were measured during a 2-hour period (from 09.00 to 11.00 hours) by telemetry at age 13 weeks. The average SBP of SHRs immunized with the ATRQβ-001 vaccine was significantly lower than that of the VLP immunization group (151±2 versus 172±2 mm Hg, P=0.002; Figure 1E).

### ATRQβ-001 Vaccination Protects Target Organs From Hypertension-Induced Damage

To assess the protective effects of ATRQβ-001 immunization in animals, the target organs were examined for hypertensive-induced injury. Examination of the SHRs by echocardiography revealed that, compared with the VLP group, the left ventricular hypertrophy in the ATRQβ-001 vaccinated group was significantly attenuated (Table). Interestingly, some parameters of the ATRQβ-001 vaccinated group were seemingly superior to that of valsartan, though there was no significant difference. Continuous Ang II–induced cardiac fibrosis in each mouse group was observed using Masson trichrome staining. The ATRQβ-001 vaccine significantly decreased the fibrosis induced by Ang II perfusion. In the VLP+Ang II group, the fibrotic area of the heart was 21.1±0.8%, whereas it was only 6.8±0.5% in the ATRQβ-001 vaccine group and valsartan group (P=0.001; Figure 2A).

### Table. ATRQβ-001 Vaccination Protects Rat Hearts From Hypertension-Induced Remodeling

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<th>Valsartan</th>
<th>ATRQβ-001</th>
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LV indicates left ventricle; BW, body weight; IVsd, diastolic interventricular septum; LVIDd, diastolic left ventricle internal diameter; LVPWd, diastolic left ventricular posterior wall; EF, ejection fraction; and FS, fractional shortening.

LV mass, IVsd, LVIDd, LVPWd, EF, and FS of spontaneously hypertensive rats in different groups were measured by ultrasonographic cardiography. The ATRQβ-001 vaccine effectively alleviated the hypertension-induced heart remodeling. All data are expressed as the mean±SE, *P<0.05 and †P<0.01 vs the virus-like particles group.

After euthanizing, the pathological changes of the heart and kidney from the SHRs in the ATRQβ-001 vaccine, VLP, or valsartan group were detected. Cardiomyocytes in the VLP group were obviously hypertrophied compared with those in the ATRQβ-001 vaccine or the valsartan group (myocyte diameter: 16.7±0.5 versus 14.0±0.4 μm, P=0.03), but no difference was observed between the ATRQβ-001 vaccine and valsartan groups (Figure 2B). For the kidneys by transmission electron microscope analysis, the basement membrane retained its integrity, and no obvious proliferation of cells in the mesangial region was observed in the ATRQβ-001 vaccine group, whereas that of the VLP group was distinct (Figure 2C). The pathological changes in the ATRQβ-001 vaccine and valsartan groups were similar. At the molecular level, compared with the VLP group, the expression levels of remodeling-related genes such as c-Jun and Brain Natriuretic Peptide and collagen type III group were downregulated, whereas Matrix Metallo Proteinase 2 and Matrix Metallo Proteinase 9 were upregulated in the ATRQβ-001 group (Figure S2 in the online-only data supplement).

### Circulating or Local RAS Are Not Activated in ATRQβ-001–Vaccinated SHRs, but AT_R Expression Is Dramatically Declined in Vaccine Group

To determine whether blockade of AT_R may lead to feedback activation of RAS, we detected the plasma renin activity and Ang II concentration in different SHR groups. The plasma renin activity in the ATRQβ-001 vaccine group was 5952.0±383.2 pmol/(h L), which was not significantly higher than that in the VLP group (5620.7±631.1 pmol/h L, P=0.509; Figure 3A). However, a significant increase in plasma renin activity was observed in valsartan groups (11 402.0±350.8 pmol/h L, P=0.02; Figure 3A). Similarly, the concentration of Ang II in the valsartan group (425.3±19.5 pmol/L) was higher than that in the VLP group (217.7±11.2 pmol/L, P=0.03), whereas no significant difference was observed between the VLP and vaccine groups (255.1±38.5 pmol/L, P=0.69) (Figure 3B).

To evaluate the change of local tissue RAS, SHRs were euthanized when the anti–ATR-001 antibody titer reached the peak. The change of concentration of Ang II in heart and kidney had no difference in 3 groups (Figure 3C). The mRNA expression of heart renin in each group was nearly undetectable, whereas the mRNA expression of kidney renin in the valsartan group was obviously higher than that of the VLP group; no difference was found between the ATRQβ-001 vaccine group and the VLP group (Figure S3). However, AT_R expression (including mRNA and protein) in the vaccine group and valsartan group was dramatically declined in heart and kidney (Figure 3D and 3E).

### Anti–ATR-001 Antibody Specifically Binds to AT_R and Effectively Inhibits Its Activation by Ang II

Results of the Western blotting and immunofluorescence assays demonstrated that the anti–ATR-001 antibody specifically bound to AT_R in rat small arterial smooth muscle cells and HEK293 cells stably expressed the human AT_R (Figure 4). No binding was observed when the antibody was neutralized by
the ATR-001 peptide (Figure 4). Complete cross-reactivity of the anti-ATR-001 antibody with the ATR-001 and ATR12181 epitopes was confirmed (Figure S5). The ATR-001-specific antibody also effectively inhibited the protein kinase C-α translocation from the cytoplasm to the periphery of the nucleus compared with the control antibody group, and this inhibitory effect was abolished when the ATR-001-specific antibody was neutralized by the ATR-001 peptide (NAT-001 group; Figure 5A).

The Ang II–induced expression of phosphorylated extracellular signal-regulated kinase 1/2 in the anti–ATR-001 preincubated group was also lower than that of the control antibody or NAT-001 group (72% decrease, \( P=0.013 \); Figure 5B, Figure S6 and S7). Furthermore, anti–ATR-001 inhibited the increase of cytosolic calcium concentration induced by Ang II (68% decrease, \( P=0.017 \); Figure 5C). No agonistic or antagonistic role was detected when stimulating the cells using the anti–ATR-001 antibody only without Ang II stimulation (Figure 5).

### Anti–ATR-001 and Ang II Do Not Competitively Bind With AT1R

Losartan competed with \(^{[125]I}\)-Ang II from binding to AT1R in a dose-dependent manner (Figure 6A). More than 95% of \(^{[125]I}\)-Ang II was displaced by losartan at the highest concentrations tested, giving a 50% inhibitory concentration (IC50) of 18.4 nmol/L, which indicated that this assay was valid for sample testing. However, no competitive binding with AT1R was detected between anti–ATR-001 and Ang II over a range of antibody concentrations (Figure 6B).

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**Figure 2.** ATRQβ-001 vaccination protects heart and kidney tissues from hypertension-induced damage. A, The fibrotic area (%) of the heart in each mouse group was calculated as the sum of all fibrous tissue areas divided by the sum of the total heart areas averaged over 6 representative fields. Bar, 50 μm. B, Diameters of cardiomyocytes were determined along the long axis in 20 representative myocytes. Bar, 50 μm (upper) and 20 μm (lower). C, Integrity of the basement membrane was intact, and no obvious proliferation of cells in the mesangial region was detected in the ATRQβ-001 vaccine group, whereas that of the VLP group was distinct. All data are expressed as the mean±SE, *\( P<0.05 \) vs the virus-like particles (VLP) group, **\( P<0.01 \) vs the VLP+angiotensin (Ang) II group.
ATRQβ-001 Vaccination Protects Against Immune-Mediated Injury

After immunizing Kyoto Wistar rats with the ATRQβ-001 vaccine, pathological changes of the heart, lung, liver, spleen, and kidney on days 7, 35, 63, and 105 were observed by light microscopy and transmission electron microscope. Compared with the control group, no obvious tissue injury and inflammatory cells infiltration were observed at any time point in any of the organs (Figure 7A and 7B, Figure S8–S11). Variations of the anti–ATR-001 antibody titer were detected in these animals, and its half-life was calculated at 14.4 days, with a 95% CI of 9.1 to 34.4 days (Figure 7C).

Discussion

The ECL2 of AT1R plays an important key role in ligand-induced receptor activation. Although the antibodies

Figure 3. Circulating or local renin-angiotensin system are not activated in ATRQβ-001–vaccinated spontaneously hypertensive rats, but AT1R expression is dramatically declined in vaccine group. Both of plasma renin activity (A) and plasma angiotensin (Ang) II concentration (B) detected after immunization with the ATRQβ-001 vaccine were not significantly elevated compared with the virus-like particles (VLP) group. C, The concentration of Ang II in heart and kidney had no difference in the 3 groups. *P<0.05 vs the VLP group. The AT1R mRNA level (D) and protein expression (E) were dramatically declined in vaccine group. The GAPDH was as internal control. *P<0.05 vs the VLP group for heart and †P<0.05 vs the VLP group for kidney.

Figure 4. Anti–ATR-001 antibody specifically binds to the angiotensin II receptor type 1 (AT1R). Western blot (A) and immunofluorescence (B) analysis in small arterial smooth muscle cells (SASMCs) and HEK293 cells stably expressing human AT1R showed that the anti–ATR-001 antibody specifically bound to AT1R. NATR indicates neutralized ATR. Bar, 20 μm.
against this ECL2 sequence discovered thus far show agonistic effects, we cannot rule out the possible existence of blocking antibodies to that region. Many researchers have attempted to identify a G protein coupling receptor antibody that can act as an agonist or an antagonist. A classic example is the detection of autoantibodies in patients with Graves’ disease that displays various functions, including activation, inhibition, or antigen-antibody binding without signal transduction activity. On the basis of the possible binding sites of Ang II with AT1R ECL2 and our previous work, the ATRQβ-001 vaccine was produced to evaluate the antihypertensive effect, and the anti-ATR-001 antibody was purified to detect the detailed blocking mechanism.

To confirm the antihypertensive effects of the ATRQβ-001 vaccine, the Ang II–induced hypertensive mouse model was first adopted. As expected, the vaccine could significantly

Figure 5. ATR-001–specific antibody effectively inhibits Ang II–induced activation. The ATR-001–specific antibody inhibited the Ang II–induced protein kinase C (PKC)–α translocation (A) and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in HEK293 cells stably expressing Ang II type 1 receptor (AT1R). C, The ATR-001–specific antibody also suppressed the elevation of intracellular calcium concentration stimulated by Ang II. No direct agonistic or antagonistic role was observed for the ATR-001–specific antibody. Con indicates control; Con-Anti, the control antibody; ATR, angiotensin II receptor type 1; NATR1, ATR-001-specific antibody neutralized by the ATR-001 peptide; Los, losartan; and Ang II, angiotensin II. All data are expressed as the mean±SE, *P<0.05 vs the control antibody group, †P>0.05 vs the losartan group. Bar, 10 μm.
decrease hypertension in the mouse model and effectively prevent Ang II–induced myocardial fibrosis. To further evaluate the antihypertensive effects, SHRs were vaccinated with ATRQβ-001 and observed for long-term period. The SBP of the ATRQβ-001 immunized SHRs obviously decreased compared with the VLP group. In the early stage after vaccination, the decrease of blood pressure was similar between the ATRQβ-001 vaccine and valsartan groups. However, after the anti–ATR-001 antibody titers decrease, the antihypertensive effect in the ATRQβ-001 vaccine group gradually receded compared with that in the valsartan group. Using these animals to assess the ATRQβ-001 vaccine for a more extended period would be a little difficult, as it has been reported that the number of T cells in SHRs decreases progressively, and T cell functions are abnormal from age week 8.19 At this time point, the blood pressure of SHRs begins to rise rapidly. Because the changes in T cells would not influence the effects of chemical drugs, we could not confidently compare the antihypertensive effects of the vaccine and valsartan in SHRs during the later stages of the experiment.

In our study, we had given the sufficient evidence that the ATRQβ-001 vaccine effectively protected the vulnerable target organs. However, the antihypertension mechanism of the ATRQβ-001 vaccine was unclear. The anti–ATR-001 antibody treatment effectively inhibited Ang II–induced protein kinase C-α translocation and extracellular signal-regulated kinase phosphorylation compared with both the control antibody and treatment with the ATR-001 peptide neutralized AT1R antibody. The anti–ATR-001 antibody also inhibited the Ang II–induced increase in intracellular transient Ca2+ concentration. For the
in vitro radiolabeled binding experiment, no competitive binding was observed between the anti–ATR-001 antibody and the agonistic Ang II. Previous binding of the Ang II or ARB to AT$_1$R did not affect the antibody bound to it (Figure S12). Furthermore, compared with the obvious RAS feedback of ARB, plasma or local RAS showed no evident increase in SHRs immunized with the ATRQβ-001 vaccine. However, just like the valsartan group, the AT$_1$R mRNA and protein expression of local tissue in vaccine group was declined significantly. Of note, FISIC (Fibrinolysis and Insulin Sensitivity in Imidapril and Candesartan) study showed that ARB may increase plasminogen activator inhibitor type 1 levels through feedback increased Ang II activating insulin-regulated aminopeptidase. No obvious RAS feedback of the ATRQβ-001 vaccine indicated that the effect of target organ protection may be better than ARB. Here, we had to take note that the AT$_1$R mRNA transcription in Figure S2A was quite high but not in Figure 3D and 3E. The reasonable explanation may be that the tissue sample was from the SHRs of which the antibody titer had nearly disappeared after days 112 (as shown in Figure S2A), and the elevation of AT$_1$R mRNA transcription was a rebound of a long-term expression inhibition of the receptor.

Fu et al reported that the AT$_1$R autoantibodies against ECL2 from malignant hypertensive patients showed agonistic effect against AT$_1$R in vitro, but no competing binding effect with AT$_1$R was observed compared with agonist or antagonist. Interestingly, the same group found 2 antibody populations against the ECL2 of M2 muscarinic receptor: 1 inhibited radiolabeled antagonist binding, whereas the other did not, although both had similar physiological effects. These antibodies were termed functional antibody by the authors. The authors pointed out that the glycosylation sites of ECL2 may be an obstacle leading to lower accessibility for antibody interactions with the receptor. In particular, 2 glycosylation residues (Asn$_{176}$, Asn$_{188}$) are present in ECL2 of AT$_1$R. The A-F-H-Y-E-S-Q epitope spans from position 181 to 187 in close proximity to the 188N site. It is worth noting that a study showed that Ang II binding with AT$_1$R increases the accessibility of Ala$_{181}$, Phe$_{183}$, and His$_{186}$. This specific conformational change is followed by AT$_1$R activation and signal transduction. The anti–ATR-001 antibody may block Ang II–induced AT$_1$R activation as a kind of functional antibody through binding to the AT$_1$R ECL2 region, which includes Ala$_{181}$, Phe$_{183}$, and His$_{186}$. Thus, the binding of anti–ATR-001 antibody may block Ang II–induced AT$_1$R activation of circulating or local RAS, including angiotensin I and Ang II, were used to immunize the animals. With the conjugation of a carrier protein, angiotensin I was shown to be antigenic and safe in animals and humans. However, neither BP nor aldosterone concentration in blood secretion was significantly modified by angiotensin I immunization in humans. The Ang II vaccine CYT-006-AngQb is a promising therapeutic vaccine based on VLP carrier. Clinical data demonstrated that vaccination with CYT-006-AngQb led to a significant reduction in SBP and no serious adverse events in hypertensive patients. However, the significant increase of Ang II in the CYT-006-AngQb vaccine may cripple the antihypertensive effect and target organ protection to some extent.

The safety of the ATRQβ-001 vaccine is another important consideration. The known types of immunological injuries are as follows: (1) immune complex deposition; (2) antibody-dependent cell-mediated cytotoxicity; and (3) activation of cytotoxic T cell against self antigens. Immune complex deposition is usually observed in the kidney, especially in the glomerular basement membrane. In our study, the kidney damage caused by immune complexes was not detected. For antibody-dependent cell-mediated cytotoxicity, still there are several controversies in terms of its onset and regulation. Overall, no visible pathological changes were detected by light microscopy. Nevertheless, the potential for antibody-dependent cell-mediated cytotoxicity to be caused by the vaccine will need to be further investigated. As the target peptide of the vaccine was only 8 amino acids in length and shorter than the minimal T cell epitope, the number of CD8$^+$ cytotoxic T cells possibly induced and activated against the ATR-001 peptide was dramatically decreased. From the results above, the ATRQβ-001 vaccine was found to be basically safe, although further assessments are needed to confirm this conclusion.

**Perspectives**

The ATRQβ-001 vaccine is a novel therapeutic method for hypertension. Compared with traditional chemical drugs, the hypertension vaccine has potentially superior advantages, including the ability to steadily lower blood pressure and greatly improve patient compliance. No significant immune-mediated damage was detected in vaccinated hypertensive and non-hypertensive animals. Additionally, no obvious feedback activation of circulating or local RAS was observed in the ATRQβ-001 vaccine group. The half-life of the antibody was determined to be 14.4 days, which is longer than any other chemical drugs presently used. These advantages of the ATRQβ-001 vaccine may provide better target organ protection. Although the animal models restricted the long-term evaluation of the vaccine compared with antihypertensive drugs, with further development it may become a promising treatment method for hypertension in humans.

**Acknowledgements**

We are thankful to Dr. Tingting Ning for the excellent technical assistance and Heping Guo for his support in our work.
Sources of Funding
This work was supported by the National Science and Technology Major Project (2009ZX09103-697), and National Natural Science Funds of China (No.30700295, No.30971246).

Disclosures
None.

References

Novelty and Significance

What Is New?
• We first provide a novel therapeutic vaccine against human AT1R for hypertension. The ATRQ1-001 vaccine significantly decreased the SBP of hypertensive animals and was similar to or, for some parameters, better than that of valsartan in protecting target organs.
• Meanwhile, the anti–AT1R-001 antibody was the first blocking antibody against AT1R ECL2, compared with the agonistic antibodies against this ECL2 discovered thus far.
• No obvious feedback activation of circulating or local renin-angiotensin system was observed in the ATRQ1-001 vaccine group.

What Is Relevant?
• The half-life of the antibody was 14.4 days, which is longer than presently used chemical drugs. The vaccine could steadily lower blood pressure, provide better target organ protection, and greatly improve patient compliance.

Summary
The ATRQ1-001 vaccine may become a promising treatment method for hypertension in humans.
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Hypertension. 2013;61:408-416; originally published online November 26, 2012; doi: 10.1161/HYPERTENSIONAHA.112.201020

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Effectiveness and Safety of a Therapeutic Vaccine Against AT1 Receptor in Hypertensive Animals

A Vaccine Against AT1 Receptor

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SUPPLEMENTAL MATERIAL

Materials and Methods
All procedures relevant to the care and use of animals in the current study conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and were approved by the Institutional Authority for Laboratory Animal Care of China.

Peptide synthesis
The ATR-001 peptide, with the sequence C-A-F-H-Y-E-S-Q corresponding to an epitope of the ECL2 of human AT1R, was synthesized by GL Ltd. (Shanghai, China). The purity was above 98% and was determined using high-performance liquid chromatography (HPLC) and mass spectrometry (MS).

Vaccine preparation
The Qβ VLP prokaryotic expression plasmid was constructed (Figure S1), expressed in BL21 Escherichia coli and purified by acidification, sedimentation of saturation ammonium sulfate, hydrophobic interaction chromatography (GE Healthcare), and gel filtration chromatography (GE Healthcare). The expressed VLP was identified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transmission electron microscope (TEM). The ATR-001 peptide was covalently conjugated to VLPs using the Sulfo-SMCC crosslinker (Pierce) to produce the ATRQβ-001 vaccine. The vaccine concentration was determined using Bradford protein assay kit (Pierce).

Vaccination of hypertensive animals and blood pressure measurement
Male BALB/c mice aged 8 wk were purchased from the experimental animal research center (Hubei province, China), whereas male SHRs aged 6 wk were from Vital River Laboratories in China (Beijing). All animals were kept in the specific-pathogen-free room in the experimental animal center (Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China). Balb/c mice (n = 10 per group) were immunized subcutaneously (s.c.) on days 0 and 14 with ATRQβ-001 vaccine or Qβ VLP formulated in aluminum hydroxide gel (Sigma), 100 μg per mouse. Another group was treated with peroral valsartan (10 mg/kg/d) (Norvatis). Vehicle group mice were injected with the same volume PBS. ATR-001-specific antibody titers were screened on days 14, 20, 27 and 36. ATR-001-specific antibody titers were screened on days 0, 7, 14, 28, 42, 56, 70, 98, and 120.
The blood pressure of animals was measured using the tail-cuff method (BP-98a, Japan). For indirect systolic blood pressure (SBP) measurements, the animals were placed in a dark chamber at 37 °C for 15 min and then transferred to a dark cage with a heating pad. The tail-cuff pressure was continuously monitored, and the signals from the pulse and pressure sensors were recorded. The SBPs were calculated from 20 readings for each animal. The SBP measurements were performed by one person who was blinded to the identities of the groups. All the measurements were performed in a quiet environment at 20-25 °C. Additionally, in SHRs, SBPs were measured by telemetry using the DSCF-08 telemetric transducer (Japan) on days 42-48 (n=4 per group, aged 13 weeks), as recommended by the manufacturer. Measurements were performed during a 2-hours period (from 0900 hours to 1100 hours), and the results were the average values of these measurements.

**Plasma renin activity (PRA) and Ang II concentration measurement**

Blood samples were collected from the jugular vein of nembutal-anesthesized rats, and then mixed with the enzyme inhibitor mixture (1ml serum in 50ul inhibitor mixture including 20ul 0.3mol/L EDTA, 10ul 0.32mol/L dimercaprol and 20ul 0.34mol/L 8-OH-quinoline sulphate). The PRA and Ang II concentration were detected according to the assay kit instruction (NIBT, Beijing) and previous description.¹

For tissue Ang II measurement, each organ was removed from decapitated SHRs within two minutes between 9 a.m and 12 a.m. Immediately after harvesting and weighing, the heart and kidney cortex were immersed in ice-cold methanol, minced, and homogenized with tissue homogenizers. The homogenates were centrifuged (4000rpm, 4°C, 15min) and the supernatants were dried overnight in a vacuum centrifuge. The dried residue was reconstituted in 1 ml radio-immunoassay (RIA) buffer and then was subjected to HPLC to separate Ang II from other substances. The tissue Ang II concentration was detected according to the assay kit instruction (NIBT, Beijing) and previous description.²

**Echocardiography**

Animals were anesthetized with 2% isoflurane and kept warm on a heated platform, and then echocardiography was performed with a Vevo 2100 high-resolution microimaging system (30 MHz, Visualsonic, Canada). Both long-axis and short-axis view of the left ventricle (LV) was obtained. Left ventricular diastolic/systolic inner diameter (LVIDd/s), interventricular septal diastolic/systolic thickness (IVSd/s), left ventricular posterior wall diastolic/systolic thickness (LVPWd/s), LV mass, LV ejection fraction (EF%), and fractional shortening (FS%) were measured.

**Morphological assessment of target organs**

After the animals were sacrificed, the heart and kidney in each animal were rapidly excised. The organs were further dissected and fixed in 4% paraformaldehyde. The fixed transverse sections of the tissues were embedded in paraffin and stained with hematoxylin-eosin (H&E) or Masson’s trichrome. For Masson’s trichrome staining, the severity of fibrosis was assessed using the percentage of the heart section with impairment compared with the overall image.
size of the heart section.\textsuperscript{3,4} Six images per mouse were chosen to be analyzed. Cardiomyocyte diameter was determined perpendicular to the outer contour of the cell membrane at the nucleus level in 20 representative myocytes of the section as described previously.\textsuperscript{3,4} A microscope eyepiece grid with magnification *200 according to the corresponding scoring system (GL6-HPIAS) was used to analyze the images. Parts of fresh renal cortex were immediately fixed in 0.25% glutaraldehyde for transmission electron microscopy (TEM).

**Quantitative real-time PCR (qRT-PCR) and tissue AT1R protein expression analysis**

Total RNA from the left ventricle tissue and kidney cortex was extracted using TRIzol reagent (Invitrogen), the procedure followed the protocol of the manufacturer. The expression of the associated marker genes Renin, AT1R, c-Fos, c-Jun, Brain Natriuretic Peptide (BNP) and collagen types I/III (Col I/III), and matrix metalloproteinases 2/9 (MMP2/9) was assessed using qRT-PCR. The first-strand cDNA was synthesized using oligo d(T) and reverse transcriptase (M-MLV, Invitrogen). qRT-PCR was performed at Step One Real-Time PCR machine (Applied Biosystems) using Platinum SYBR qPCR superMix-UDG (Invitrogen). Primers were showed in Table S1.

The AT1R protein expression level of heart and kidney was detected by Western blotting. After weighing and homogenizing in ice-cold protein extraction buffer (Pierce) containing a protease inhibitor cocktail (Roche Applied Science), the homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C. The protein concentration of the supernatant was confirmed using the BCA assay kit (Pierce). The levels of both AT1R and GAPDH were detected using the anti-AT1R polyclonal antibody (1:500, Sigma) and anti-GAPDH monoclonal antibody (1:1000, Cell Signaling Technology) respectively.

**Antibody preparation**

The antibodies were from sera of 12-week-old AT1 vaccinated rats. The antibody titers were determined by ELISA, and the sera were purified using protein A affinity chromatography (Bio-Rad) and additionally purified using epitope-linked gel affinity chromatography (GE Healthcare). The “control antibody” was purified from rats that were immunized with VLP only. In all of the experiments, the final working concentration of the antibody was 1.0 μg/ml. The peptide neutralization antibody was produced by the co-incubation of 1 mg of peptide and 1 mg of the antibody in phosphate-buffered solution (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na\textsubscript{2}HPO\textsubscript{4}, and 1.4 mmol/L KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) for 8 h at 4 °C. The peptide neutralization antibody was termed the NATR-001 antibody.

**Cell culture**

Small arterial smooth muscle cells (SASMCs) were obtained from the superior mesenteric arteries of Sprague-Dawley rats (~250 g).\textsuperscript{5} Superior mesenteric arteries were isolated from male Sprague-Dawley rats that had been injected with buprenorphine hydrochloride (0.2mg/kg) subcutaneously for analgesia and euthanized by peritoneal injection of sodium pentobarbital solution (150 mg/kg). The superior mesenteric arteries were separated and dissociated from the adventitial connective tissue. In the first step of digestion, the vessel
tissue was incubated in enzyme solution I (1.4 mg/ml collagenase, 0.5 mg/ml elastase, 1 mg/ml soybean trypsin inhibitor, and 5 mg/ml crystallized bovine albumin) for 10 min at 37 °C to separate the medial layer. In the second step, the medial layer was digested by incubation in enzyme solution II (2 mg/ml collagenase, 0.5 mg/ml elastase, 1 mg/ml soybean trypsin inhibitor, and 5 mg/ml crystallized bovine albumin) for 60 min to obtain single SASMCs, which were identified using the monoclonal antibody against smooth muscle α-actin (Millipore). SASMCs were cultivated in Dulbecco’s modification of Eagle’s medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS, Gibco). Human embryonic kidney 293 cells (HEK293, ATCC) were stably transfected with a pCDNA3.1 (+) vector expressing AT1R, which was constructed by our lab and cultured in DMEM containing 20% FBS. Rat myocardium H9c2 cells were from ATCC and cultured in DMEM containing 20% FBS.

**Antibody identification**

Western blotting and immunofluorescence were used to demonstrate that the anti-ATR-001 antibody specifically bound to AT1R. Rat SASMCs or HEK293 cells that stably expressing human AT1R were harvested. The total proteins were extracted using protein extraction buffer (Pierce) that contained a protease inhibitor cocktail (Roche Applied Science). The anti-ATR-001 antibody was used as primary antibody. Specific bands were detected using the chemiluminescence assay (ECL detection reagents, Pierce). For immunofluorescence, cells were fixed with 4% paraformaldehyde at -20 °C for 5 min and incubated with the anti-ATR-001 antibody overnight at 4 °C. An Alexa 488 Fluor-conjugated antibody (1:200, Invitrogen) was used as the secondary antibody. Nuclei were stained with 4’, 6-diamidino-2-Ang II Inylindole (DAPI). The preparation was imaged using an Olympus FV500 confocal microscope (Japan).

**Immunocytochemistry**

HEK293 cells that stably expressing human AT1R were divided into 5 groups as follows. The control group was untreated, while the other four groups were pre-incubated with either the control antibody, losartan (100 µmol/L, Sigma), ATR-001-specific antibody or peptide neutralized (NATR-001) antibody for 40 min before stimulation with 100 µmol/L Ang II (Sigma). To demonstrate the direct effects of the antibody against AT1R, another group of cells was stimulated with the ATR-001-specific antibody directly without Ang II stimulation. PKC-α translocation was detected as previously described. The HEK293 cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol at -20 °C for 5 min. After being blocked with 2% BSA in PBS for 60 min, the cells were incubated for 1 h with anti-PKC-α antibody (1:100, Millipore). The cells were washed three times with PBS and incubated for 40 min with the Alexa-488 conjugated secondary antibody, which was diluted in 1% BSA/PBS (1:200, Invitrogen). An Olympus FV500 confocal imaging system (Japan) was used to scan samples. With the exception of fixation and permeation, all the experiments herein were carried out at room temperature.
Phosphorylated ERK1/2 levels
The level of ERK1/2 phosphorylation was detected by Western blotting in HEK293 cells expressing AT1R, rat SASMCs and H9c2 cells. The design of the groups was identical to that for the PKC-α translocation experiment. Cells were scraped on ice and washed twice with cooling PBS. After collecting the cells by centrifugation at 3000 rpm for 10 min, the cells were transferred into protein extraction buffer (Pierce) containing a protease inhibitor cocktail (Roche Applied Science) and a phosphatase inhibitor cocktail (Roche Applied Science). After a 30-min lysis procedure, the total proteins were extracted by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentration was confirmed using the BCA assay kit (Pierce). The levels of both phosphorylated and total ERK1/2 were detected using the phosphorylated-ERK1/2 (P-ERK1/2) monoclonal antibody (1:1000, Cell Signaling Technology) and total ERK1/2 monoclonal antibody (1:1000, Cell Signaling Technology) respectively.

Intracellular Ca^{2+} concentration measurement
HEK293 cells that stably expressing human AT1R were plated onto chamber slides (Nalge Nunc International) before measuring the cytosolic Ca^{2+} concentration similar to the previous description. The cells were divided into five groups: the control antibody; anti-ATR-001; NATR-001 antibody; the losartan group, following the Ang II stimulation, and another group, which was stimulated with anti-ATR-001 antibody directly. The former three groups were preincubated with the control antibody, anti-ATR-001, or NATR-001 antibody, respectively, for 40 min at 37 °C. Then, the cells were washed twice with PBS, and the solution was replaced by 0.7 ml of DMEM with 20% FBS, Fluo-3-AM (final concentration of 5 µmol/L, Sigma), and Pluronic F127 (final concentration of 0.02%, Sigma-Aldrich). The cells were loaded in the dark, and the loading solution was aspirated after 30 min. Cells were gently washed twice and incubated in 0.3 ml of HEPES-buffered Hank’s salt solution (HBSS, pH 7.4) for 30 min. The measurement of the cytosolic Ca^{2+} concentration was performed using an Olympus FV500 confocal system (Japan). A total of 10 µl Ang II (final concentration of 100µmol/L) was added to the chamber, and the fluorescence intensity was recorded. The cells in the losartan group were preincubated with losartan (100 µmol/L) for 10 min after being loaded with Fluo-3-AM and then stimulated with 100 µmol/L Ang II. All the measurements were performed at room temperature for three independent experiments.

AT1R Radioligand binding assays
The AT1R binding assay was performed as described previously. Briefly, HEK293 cells stably expressing human AT1R in 12-well plates were cultured in DMEM supplemented with 0.5% FBS for 2 h. The cells pretreated with different dose of anti-ATR-001 for 40 min at 37°C, and then incubated for 30 min with 0.2 nmol/L of [^{125}I]-Ang II (Perkin Elmer). Cells were then washed three times with ice-cold PBS containing 0.1% BSA and lysed in 0.5 mol/L NaOH. The radioactivity count of the lysate was measured by γ-counter.

Vaccination of WKYs and antibody half life measurement
WKYs were immunized s.c. on days 0, 14, and 28 with 300 μg of the ATRQβ-001 vaccine. Anti-ATR-001 titers were screened on days 0, 7, 14, 28, 35, 42, 49, 63, 77 and 105. The animals were sacrificed on days 7, 35, 63 and 105 for histological evaluation. For immunohistochemical staining, the anti-rat CD14 antibody (ABBIOTEC) and anti-rat CD19 antibody (R&D system) were used. The antibody half-life was obtained by fitting data to a single exponential decay, and the confidence interval was calculated using GraphPad Prism.

**Statistical Analysis**

Data are presented as means ± S.E. Analysis of variance (ANOVA) was used to compare variables among groups. One-way repeated measures ANOVA was used for analysis of the SBP. Multiple linear regression was used to analyze the relationship between SBP variation and the ATR-001-specific antibody titer. $P<0.05$ was accepted as significant.
References

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Figure S1 Vaccine preparation

(A) The capsid gene of Qβ bacteriophage was cloned into pET28a vector digested by Nco I and Xho I. (B) Qβ virus like particle (VLP) was synthesized by prokaryotic expression system. The left figure showed the expression of Qβ VLP was induced by IPTG. The middle figure showed the purification process and the eluted protein was identified by SDS-PAGE. The right figure showed the peptide ATR-001 conjugated to the VLP. (C) Qβ VLP was stained by uranylgacetate and identified by TEM.
Figure S2

Expression levels of genes associated with cardiac remodeling were analyzed by qRT-PCR. (A) The \textit{AT1R} gene was significantly elevated in the ATRQβ-001 vaccine group. (B, C and D) The hypertrophy marker genes \textit{c-Jun} and \textit{BNP} were downregulated in the ATRQβ-001 vaccine group. (E) No obvious change of \textit{Col I} was detected in both the ATRQβ-001 vaccine group and valsartan group. (F) The expression of fibrosis marker gene \textit{Col III} was decreased in the ATRQβ-001 vaccine group. (G and H) Levels of \textit{MMP2/9} were elevated in the ATRQβ-001 vaccine group. The levels of target genes relative to those of the VLP group were calculated by the $2^\Delta\Delta C_t$ method, normalized by GAPDH expression. \textit{BNP}, Brain Natriuretic Peptide; \textit{Col I/III}, collagen types I/III; \textit{MMP2/9}, matrix metalloproteinases 2/9. All data are expressed as the mean ± S.E., * $P<0.05$ and ** $P<0.01$ vs. the VLP group, † $P<0.05$ vs. the valsartan group.
Figure S3. The expression of renin mRNA in kidney was increased in the valsartan group but not in the ATRQβ-001 vaccine group. The renin mRNA expression relative to VLP group was significant elevated in the valsartan group but not in the ATRQβ-001 vaccine group. ** P<0.01 vs. VLP group
Figure S4

AT1R was stably expressed on HEK 293 cell line

AT1R was stably expressed on transfected HEK 293 cells but not the native cells.
Figure S5

Figure S5 Cross-reactivity of Anti-ATR-001 between epitope ATR-001 and ATR12181

ATR-001 and ATR12181 were used as the epitopes to demonstrate the cross-reactivity of anti-ATR-001. The antibody was diluted as 1:1000 and used as the in ELISA. The anti-ATR-001 was also co-incubated with the peptides ATR-001, and then the titers of anti-ATR-001 were detected.
Figure S6

**Figure S6 ATR-001-specific antibody inhibited the Ang II-induced ERK1/2 phosphorylation in SASMCs**

The ATR-001-specific antibody inhibited the Ang II-induced ERK1/2 phosphorylation in SASMCs. No direct agonistic or antagonistic role was observed for the ATR-001-specific antibody. Con, the control antibody; Anti-ATR, the anti-ATR-001 antibody; Los, losartan; Ang II, angiotensin II. All data are expressed as the mean ± S.E, **P<0.01 vs. the control antibody + Ang II group.**
Figure S7

Figure S7 ATR-001-specific antibody inhibited the Ang II-induced ERK1/2 phosphorylation in H9c2 cells

The ATR-001-specific antibody inhibited the Ang II-induced ERK1/2 phosphorylation in H9c2 cells. No direct agonistic or antagonistic role was observed for the ATR-001-specific antibody. Con, the control antibody; Anti-ATR, the anti-ATR-001 antibody; Los, losartan; Ang II, angiotensin II. All data are expressed as the mean ± S.E, ** P<0.01 vs. the control antibody +Ang II group, † P>0.05 vs. the control antibody group.
Figure S8 Pathological assessment of organs from immunized SHRs
No obvious change of kidneys and hearts was observed under light microscope using Masson’s trichrome staining. Bar, 50 μm.
Figure S9 Pathological assessment of organs from immunized KYW
WKYs were immunized subcutaneously (s.c.) on days 0, 14 and 28 with ATRQβ-001 vaccine. No immune-mediated damage was detected in heart, lung, liver, kidney and spleen on days 7, 35 and 63. Bar, 50 μm.
Figure S10 Pathological assessment of kidneys from immunized KYW by TEM
WKYs were immunized subcutaneously (s.c.) on days 0, 14 and 28 with ATRQβ-001 vaccine. No obvious kidney damage was observed under TEM on days 7, 35 and 63.
No obvious infiltration of macrophages or other immune cells were observed in the heart, kidney and liver from immunized KYW.

WKYs were immunized subcutaneously (s.c.) on days 0, 14 and 28 with ATRQβ-001 vaccine. No obvious infiltration of macrophages or other immune cells were observed in heart, liver and kidney on days 63. Bar, 10 μm.
Figure S12

Figure S12 Prior binding of AngII or losartan to AT1R did not affect the Anti-ATR-001 binding to the AT1R
The HEK293 cells expressed the AT1R were incubated with different concentration of losartan or Ang II from 10µmol/L to 100µmol/L for 30 min. Anti-ATR-001 Antibody was added into the solution and immunofluorescence was used to identify the binding between Anti-ATR-001 Antibody and AT1R. The binding capability of Anti-ATR-001 antibody to AT1R was not affected by the losartan or Ang II under different concentration. Bar, 20 µm