Angiotensin II Type 1 Receptor Antisense Gene Therapy Prevents Altered Renal Vascular Calcium Homeostasis in Hypertension

Craig H. Gelband, Phyllis Y. Reaves, Jenafer Evans, Hongwei Wang, Michael J. Katovich, Mohan K. Raizada

Abstract—Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) homeostasis regulates vascular smooth muscle tone, and alteration in [Ca\(^{2+}\)\(_i\)] handling is associated with the development and establishment of hypertension. We have previously established in the spontaneously hypertensive rat (SHR) that virally mediated delivery of angiotensin II type 1 receptor antisense (AT\(_1\)R-AS) prevents the development of high blood pressure and some pathophysiology associated with hypertension for 120 days. In light of this, our objectives in this study were to determine whether AT\(_1\)R-AS gene therapy (1) could have a longer duration in the prevention of hypertension and (2) would attenuate the alterations in renal vascular Ca\(^{2+}\) homeostasis and therefore vasoconstriction, characteristics of hypertension. Intracardiac delivery of AT\(_1\)R-AS in neonates prevented the development of hypertension in SHR for at least 210 days. At this time, untreated SHR renal resistance arterioles showed a significantly enhanced contractile response to KCl and angiotensin II (Ang II) when compared with normotensive Wistar-Kyoto rats. In addition, L-type Ca\(^{2+}\) current density and Ang II–dependent increases in [Ca\(^{2+}\)\(_i\)] were significantly increased in cells dissociated from renal resistance arterioles of the untreated SHR. AT\(_1\)R-AS treatment prevented all of the above vascular alterations associated with the hypertensive state in SHR. Finally, Western blot analysis of L-type Ca\(^{2+}\) channel (\(\alpha\)\(_{1C}\)) protein levels in renal resistance arterioles of untreated SHR showed no significant difference when compared with control. These results are novel and demonstrate that viral-mediated delivery of AT\(_1\)R-AS not only attenuates the development of hypertension on a long-term basis but prevents changes in renal vascular Ca\(^{2+}\) homeostasis associated with the disease. (Hypertension. 1999;33[part II]:360-365.)

Key Words: angiotensin II ■ arterioles ■ calcium, intracellular ■ Ca\(^{2+}\) current ■ excitation-contraction coupling ■ gene therapy

Essential hypertension is characterized by normal cardiac output and an increase in total peripheral resistance.\(^1\) Hypertension is one of the most important risk factors for stroke, congestive heart failure, myocardial infarction, end-stage renal diseases, and peripheral vascular disease.\(^2\) Studies from the last 2 decades have established that both circulating and tissue renin-angiotensin systems (RAS) are important, that their coordinated interaction is essential in the regulation of blood pressure, and that they play a key role in the development, establishment, and maintenance of hypertension.\(^2,5,6\)

The relevance of the RAS to blood pressure control is further supported by reports that various genes that encode renin, angiotensinogen, angiotensin-converting enzyme (ACE), and the angiotensin II type 1 receptor (AT\(_1\)R) have been associated with hypertension in both human and animal models.\(^7,9\) Additionally, interruption in the expression of the RAS attenuates high blood pressure and other pathophysiological aspects of hypertension.\(^4,10,11\) In fact, blockade of the RAS has become a well-accepted treatment for Ang-dependent hypertension and congestive heart failure.\(^11\) Because ACE inhibition and AT\(_1\)R blockade are standard means to treat hypertension and because AT\(_1\)R encoding gene polymorphism is coupled with hypertension in both humans and in animal models of hypertension,\(^6,9\) it would appear only logical that AT\(_1\)R is an important target in the management of high blood pressure. Although major strides have been made in developing drugs that interfere with either angiotensin II (Ang II) formation or its action toward the management of Ang-dependent hypertension, there is neither a long-term preventive measure nor a cure for this disease.

The most widely used animal model for studying human essential hypertension is the spontaneously hypertensive rat (SHR). Pharmacological intervention has been relatively
successful in normalizing the elevation in blood pressure associated with hypertension. However, the assumption that reduction of blood pressure will totally reverse hypertension-induced pathophysiological changes remains unclear. Several animal studies suggest that gene therapy would be useful in the treatment of hypertension. Olivero et al.\(^ {12} \) have shown that disruption of AT\(_R\) expression in mice causes blunting of pressor responses of Ang II. Phillips et al.\(^ {13} \) have used antisense oligonucleotides for the AT\(_R\) in the brain to reduce high blood pressure in SHR on a short-term basis. Recently, we have established that retrovirally mediated delivery of AT\(_R\) antisense (AT\(_R\)-AS) attenuates the development of high blood pressure on a long-term basis (up to 120 days) as well as prevents some of the pathophysiological alterations observed with the disease (eg, endothelial dysfunction, decreased K\(^+\) channel activity, increased heart weight, and increased cardiac and vascular fibrosis and necrosis) while having no inflammatory response.\(^ {14} \) However, a more important pathophysiological alteration, the prevention of changes in cellular Ca\(^{2+}\) handling and therefore the level of vascular tone, has yet to be investigated.

Alterations in cellular Ca\(^{2+}\) homeostasis using \(^{45}\)Ca\(^{2+}\) flux, fluorometric, and patch-clamp measurements in large conduit SHR arteries and cells have been described.\(^ {15} \) Renal transplant studies have suggested that the genetic defect in a model of the disease (eg, endothelial dysfunction, decreased K\(^+\) channel activity, increased heart weight, and increased cardiac and vascular fibrosis and necrosis) while having no inflammatory response.\(^ {14} \) However, a more important pathophysiological alteration, the prevention of changes in cellular Ca\(^{2+}\) handling and therefore the level of vascular tone, has yet to be investigated.

**Methods**

**Preparation of Viral Particles Containing AT\(_R\)-AS**

A retroviral vector, LNSV, was used to deliver AT\(_R\)-R-AS into the rats as previously described.\(^ {14} \) Briefly, the AT\(_R\)-R-AS was cloned in the LNSV vector. It was transfected to packaging cell line PA317 (American Type Culture Collection). After selection by neomycin, the medium containing viral particles that expressed AT\(_R\)-R-AS (LNSV-AT\(_R\)-R-AS) was collected and used for all animal experiments. Viral particles that did not contain AT\(_R\)-R-AS (LNSV) were also prepared by the above protocol and used as a control. The AT\(_R\)-R-AS is believed to be specific for the AT\(_R\) because delivery of a 95% survival rate at 24 to 48 hours after viral administration. The AT\(_R\)-R-AS was electrophoresed in 4% to 15% SDS–polyacrylamide gel and analyzed (Micro-Med) essentially as described.\(^ {14} \) All animal protocols are in accordance with the University of Florida institutional guidelines (IACUC approval 4138 and 5236).

**Smooth Muscle Cell Isolation**

Renal vascular smooth muscle cells were dissociated as previously described.\(^ {14,17} \) Male rats were killed by decapitation, and the right and left kidneys were removed and placed in cold oxygenated (95% O\(_2\)-5% CO\(_2\)) physiological saline solution (PSS). Renal resistance arterioles were identified as the fifth to sixth branch distal to the renal artery, cut into small pieces, and subsequently resuspended in Ca\(^{2+}\)-free PSS digestion buffer for 20 to 30 minutes at 37°C. The Ca\(^{2+}\)-free PSS digestion buffer contained (in mg/15 mL) collagenase (151 U/mg Worthington Biochemical Corp) 4.5, bovine serum albumin (BSA) 30, trypsin inhibitor 30, ATP (sodium salt) 1.7, and protease (type XXIV, Sigma Chemical Co) 1.5. After the digestion, the pieces were gently triturated until a large number of elongated smooth muscle cells were observed. The isolated cells were collected and stored at 4°C until use.

**Current Recording and Analysis**

Single cells were voltage-clamped, and membrane currents were measured with the whole-cell patch-clamp technique.\(^ {14,20} \) Data analysis was performed with pCLAMP 6.0 software (Axon Instruments). All experiments were performed at room temperature. For whole-cell voltage-clamp experiments examining Ca\(^{2+}\) currents, the bath solution contained (in mmol/L) CaCl\(_2\) 1.8, d-glucose 5.5, TEA 5.0, and HEPES 10 (pH 7.4 with CsOH). The pipette solution for the whole-cell experiments contained (in mmol/L) CsCl 140, MgCl\(_2\) 0.5, EGTA 10, ATP (magnesium salt) 5.0, and HEPES 5.0 (pH 7.2 with CsOH).

**Western Blot Analysis**

Renal resistance arterioles were snap-frozen with liquid nitrogen. To isolate proteins, 0.1 g of tissue was homogenized in 2.0 mL of cold Tris-HCl buffer containing 2.5 mg/mL leupeptin and 4 mg/mL calpain inhibitors, pH 7.4. The suspension was centrifuged at 30,000g for 20 minutes at 4°C. The resulting pellet was resuspended in the above solution and centrifuged. This pellet was resuspended in 1.2% digitonin, 300 mmol/L KCl, 150 mmol/L NaCl, 10 mmol/L NaPO\(_4\), peptatin A (0.1 \(\mu\)g/mL), leupeptin (0.1 \(\mu\)g/mL), aprotinin (0.1 \(\mu\)g/mL), AESFB (0.02 mmol/mL), benzamidine (0.01 mg/mL), and calpain (0.8 \(\mu\)g/mL), pH 7.4. The protein concentration in the supernatant was determined by Bio-Rad assay using BSA for the standard curve.

**Immunoblotting**

For immunoblotting, 20 \(\mu\)g protein was denatured with Laemmli’s sample buffer in a boiling water bath for 3 minutes. Each mixture was electrophoresed in 4% to 15% SDS–polyacrylamide gel and...
transferred onto nitrocellulose. The membrane was blocked with 10% nonfat dried milk in PBST-BSA for 1 hour followed by incubation overnight at 4°C with either anti-β-actin (1:100, Sigma) or anti-αCa2+ channel protein (1:1000, Alamone Labs). Proteinnbound antibody was detected by incubation of the membrane with horseradish peroxidase–labeled secondary antibody (1:50 000, Jackson ImmunoResearch Laboratory), enhanced by chemiluminescent assay reagents, and recognized by exposure to film.

Statistics

Results are expressed as mean±SEM. Statistical significance was evaluated using repeated-measures ANOVA and Student’s t test for unpaired data. Differences were considered significant at P<0.05. Membrane currents were measured from the zero current level and normalized to cell capacitance.

Results

Effect of AT1R-AS Treatment on Blood Pressure

A single intracardiac injection of LNSV-AT1R-AS to neonatal rats prevented the increase in blood pressure exclusively in SHR for at least 210 days after injection (Figure 1). The mean blood pressure was 36.6% lower in SHR treated with LNSV+AT1R-AS into neonatal SHR prevented an increase in blood pressure. Data are expressed as mean±SE (n=6; *P<0.01 compared with WKY, **P<0.01 compared with SHR).

Effect of LNSV-AT1R-AS Treatment on Voltage and Receptor-Dependent Renal Arteriolar Vascular Reactivity

Alterations in vascular contractile response are known to exist in SHR, but no one has investigated specifically the role of [Ca2+]i, in small arterioles of the kidney, which play a significant role in regulating peripheral resistance.1 LNSV-AT1R-AS treatment prevented the alterations in voltage- and receptor-dependent renal arteriolar vascular reactivity measured in SHR (Figure 2). Enhanced contractile responses to both KCl (Figure 2A) and Ang II (Figure 2C) were observed in the SHR. A significant leftward shift in the KCl and Ang II concentration-response relationships was observed in SHR when compared with WKY controls (Figure 2A and 2C). The media effective concentrations (EC50) for KCl and Ang II were 11.1±2.1 mmol/L and 19.7±2.8 mmol/L (n=24 rings from 6 animals) in the untreated SHR and 36.6±3.4 mmol/L and 76.4±3.3 mmol/L (n=24 rings from 6 animals) in the WKY, respectively. LNSV-AT1R-AS prevented the shift in the EC50 for both KCl and Ang II (Figure 2B and 2D). The EC50 for KCl and Ang II in the LNSV-AT1R-AS–treated SHR was 39.1±4.2 mmol/L and 68.7±4.2 mmol/L (n=24 rings from 6 animals), which was not significantly different from that of the WKY. Finally the EC50 for KCl and Ang II in the LNSV-treated SHR was 21.1±3.8 mmol/L and 17.7±2.1 mmol/L (n=24 rings from 6 animals), which was not significantly different from that of the untreated SHR.

Effect of LNSV-AT1R-AS on L-Type Ca2+ Current Density

It has been previously demonstrated that L-type Ca2+ current is increased in large conduit arteries of the SHR.15 Figure 3 illustrates the characteristics of L-type Ca2+ current in single cells isolated from renal resistance vessels of WKY and SHR. During voltage step depolarizations in the presence of block-

Figure 1. Effect of LNSV-AT1R-AS treatment on mean blood pressure in WKY and SHR. Five-day-old WKY and SHR treated with PSS, LNSV, and LNSV+AT1R-AS were allowed to mature for at least 210 days. Mean blood pressure was measured through catheters implanted in the carotid artery. A single injection of LNSV+AT1R-AS into neonatal SHR prevented an increase in blood pressure. Data are expressed as mean±SE (n=6; *P<0.01 compared with WKY, **P<0.01 compared with SHR).

Figure 2. Mean concentration-response relationship to KCl and Ang II in rat renal resistance arterioles. The KCl (A and B) and Ang II (C and D) concentration-response curve was shifted to the left in SHR and SHR+LNSV compared with WKY and SHR+AT1R-AS. This shift was prevented with delivery of AT1R-AS. Data are presented as mean±SE (n=24 rings from 6 animals).

Effect of LNSV-AT1R-AS Gene Therapy in SHR
Characteristics of L-type Ca$^{2+}$ current in single vascular smooth muscle cells isolated from rat renal resistance arteries. A. The Ca$^{2+}$ current was increased in cells from SHR and SHR+LNSV when compared with WKY. Delivery of AT,R-AS prevented this increase. The membrane capacitances of all 4 cells were similar (WKY, 27 pF; SHR, 29 pF; SHR+AT,R-AS, 28 pF; SHR+LNSV, 28 pF). B. There was no significant difference in current density of the cells from the WKY (n=21 cells from 6 animals) and SHR+AT,R-AS (n=18 cells from 6 animals). However, the Ca$^{2+}$ current density for both the peak and sustained components was significantly greater in the SHR (n=25 cells from 6 animals) and SHR+LNSV (n=20 cells from 6 animals).

AT,R-AS Gene Delivery Does Not Affect Ca$^{2+}$ Current Kinetics

<table>
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<tr>
<th>Species and Treatment</th>
<th>Activation</th>
<th>Inactivation</th>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2}$, mV</td>
<td>Slope</td>
</tr>
<tr>
<td>WKY</td>
<td>-1.5±0.6</td>
<td>7.8±0.3</td>
</tr>
<tr>
<td>SHR</td>
<td>-0.9±1.0</td>
<td>8.1±0.4</td>
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<tr>
<td>SHR+AT,R-AS</td>
<td>-1.3±0.8</td>
<td>7.7±0.2</td>
</tr>
<tr>
<td>SHR+LNSV</td>
<td>-1.4±0.4</td>
<td>8.2±2.1</td>
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Boltzmann function, membrane potential at which one-half activation or inactivation ($V_{1/2}$) or the slope of the Boltzmann function was unaffected in cells from the WKY. SHR, SHR+LNSV, or SHR+AT,R-AS. Finally, we examined whether there was an increase in Ca$^{2+}$ channel protein density in the renal vasculature of the SHR. Western blot analysis (Figure 4) using an antibody that recognized $\alpha_{1C}$ Ca$^{2+}$ channel proteins illustrated that when normalized to $\beta$-actin, there was no increase in the number of L-type Ca$^{2+}$ channels in the SHR renal resistance arterioles when compared with those in the WKY. Administration of LNSV-AT,R-AS did not alter these data.

Effect of LNSV-AT,R-AS on Voltage- and Receptor-Dependent Changes in [Ca$^{2+}$]$_i$

Because the KCl and Ang II concentration-response relationship was shifted to the left in the SHR and this was prevented in the SHR treated with LNSV-AT1R-AS, we wanted to examine next whether alterations in intracellular Ca$^{2+}$ handling would be prevented using LNSV-AT,R-AS gene therapy. We have previously shown that basol and Ang II–stimulated increases in [Ca$^{2+}$]$_i$ were greater in renal arteriolar cells of the SHR when compared with WKY.17 Figure 5 shows the change in [Ca$^{2+}$]$_i$ when renal resistance artery cells were challenged with KCl (30 mmol/L, Figure [fig 5A]) or Ang II (30 nmol/L, Figure [fig 5B]). These doses were chosen because they are approximately 50% effective. Both KCl and Ang II significantly increased [Ca$^{2+}$]$_i$ in renal arteriolar cells of the SHR when compared with WKY cells.

Figure 3. Characteristics of L-type Ca$^{2+}$ current in single vascular smooth muscle cells isolated from rat renal resistance arteries. A. The Ca$^{2+}$ current was increased in cells from SHR and SHR+LNSV when compared with WKY. Delivery of AT,R-AS prevented this increase. The membrane capacitances of all 4 cells were similar (WKY, 27 pF; SHR, 29 pF; SHR+AT,R-AS, 28 pF; SHR+LNSV, 28 pF). B. There was no significant difference in current density of the cells from the WKY (n=21 cells from 6 animals) and SHR+AT,R-AS (n=18 cells from 6 animals). However, the Ca$^{2+}$ current density for both the peak and sustained components was significantly greater in the SHR (n=25 cells from 6 animals) and SHR+LNSV (n=20 cells from 6 animals).

Figure 4. Western blot analysis of $\alpha_{1C}$ in cells from renal resistance arterioles. A representative Western blot of L-type Ca$^{2+}$ channel protein is shown. No change was evident in the amount of $\alpha_{1C}$ Ca$^{2+}$ channel protein in any treatment group when normalized to $\beta$-actin protein level (n=6).
AT₁R-AS Gene Therapy in SHR

The kidneys, which exquisitely control blood pressure and extracellular fluid volume and ultimately renal blood flow, participate in the development of essential hypertension. The results of earlier renal transplant studies in humans and animals suggest that in a proportion of hypertension cases there exist structural or functional alterations in the kidney. An elevation in renal vascular tone and therefore renal vascular resistance exists in hypertension; this elevation may be due to a number of factors including (1) an enhanced contractile sensitivity to vasoactive agonists, (2) an impaired endothelium-dependent relaxation, (3) an increased Ca²⁺ transport across vascular smooth muscle membranes, (4) an altered ion channel activity in vascular smooth muscle, and (5) smooth muscle hypertrophy or hyperplasia.

One fundamental reason why renal vascular resistance may be increased in hypertension is an altered ion channel function in vascular smooth muscle cells. Tonic changes in membrane potential and thus Ca²⁺ influx regulate contractile tone of resistance arterioles. A number of reports show that Ca²⁺ current density is increased in the SHR model of hypertension. However, these studies have been in conduit arteries, which play a less significant role in the regulation of peripheral resistance. Here we show that not only is Ca²⁺ current density increased in the SHR, but there is no change in the level of Ca²⁺ channel protein in renal resistance vessels. These data suggest that there may be an altered intracellular regulation of L-type Ca²⁺ channels that may underlie the enhanced Ca²⁺ influx in vascular smooth muscle cells and increases in vascular resistance observed in the kidney in hypertension. It is possible that an increase in Ca²⁺ influx could tend to load the sarcoplasmic reticulum with more Ca²⁺, enabling more to be released on challenge by a physiological stimulus. Here we show that this altered Ca²⁺ handling by the sarcoplasmic reticulum can be prevented using AT₁R-AS. This prevention would underlie the shift of the concentration-response curve back to normal with AT₁R-AS treatment. Physiologically, this would tend to decrease the amount of vascular tone in the kidney for a given concentration of circulating Ang II.

Finally, cardiovascular ultrastructural changes are a major risk factor for morbidity and mortality in hypertension, and Ang II has been speculated to play a role in the increased vascular hypertrophy and/or hyperplasia in hypertension. Could gene therapy delivery systems interrupting the RAS be used to control the vascular remodeling/hypertrophy seen in various forms of hypertension? We have previously shown that AT₁R-AS treatment could prevent the observed increase in perivascular fibrosis and ventricular hypertrophy in the SHR. Liao et al. have demonstrated that receptor-dependent stimulation of a Ca²⁺-dependent PKC isoform activates mitogen-activated protein kinase (MAPK) and thus regulates vascular smooth muscle cell growth. The above associated complications occur on a long-term basis, and their cellular mechanisms of action appear to be a Ca²⁺-dependent process. Therefore, interference in the RAS using gene therapy may be an important pharmacological target to block vascular ultrastructural and remodeling changes in hypertension.

Figure 5. KCl- and Ang II–mediated changes in [Ca²⁺]. Both KCl (A) and Ang II (B)–mediated increases in [Ca²⁺] were significantly greater in SHR and SHR + LNSV than in WKY (n=162 cells from 4 animals; *P<0.01). AT₁R-AS prevented the alteration in both KCl- and Ang II–mediated increases in [Ca²⁺] (n=134 cells from 4 animals; **P<0.01).
Is antisense gene therapy targeting the RAS a therapeutic step forward? In short, the answer is yes. It results in the prevention of the increase in mean blood pressure and the associated pathophysiological impairments in hypertension. It also offers an alternative to the compliance problem and complications of vascular and target-organ injury. Finally, AT1R-AS therapy does not produce a significant increase in plasma Ang II levels compared with losartan, the AT1R antagonist. Therefore, AT1R-AS gene delivery and therapy does have prolonged antihypertensive effects without the possible adverse side effects produced by traditional pharmacological therapies. One caveat of the approach taken in this study is that AT1R-AS gene delivery prevents the pathophysiological complications occurring with the development of hypertension. Moreover, this type of gene delivery/therapy depends on the identification of genetic determinants of hypertension or on the demonstration of reliable prehypertensive risk factors before it can be used in human trials. Another approach currently being investigated in our laboratory is to develop the next generation of viral vectors, which would increase the feasibility of delivering RAS-relevant genes into the adult SHR to reverse pathophysiological complications occurring with the development of hypertension. Preliminary studies show that delivery of RAS-relevant antisense into the adult SHR reverses the increase in blood pressure as well as the differences in excitation-contraction coupling observed in hypertension. In summary, gene therapy holds promise in single-dose, long-term treatment for hypertension.

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