Permanent Cardiovascular Protection From Hypertension by the AT$_1$ Receptor Antisense Gene Therapy in Hypertensive Rat Offspring

Phyllis Y. Reaves,* Craig H. Gelband,* Hongwei Wang,* Hong Yang, Di Lu, Kathleen H. Berecek, Michael J. Katovich, Mohan K. Raizada

Abstract—Our previous studies have demonstrated that the introduction of angiotensin II type I receptor antisense (AT$_1$R-AS) cDNA by a retrovirally mediated delivery system prevents the development of hypertension in the spontaneously hypertensive rat (SHR), an animal model for primary hypertension in humans. These results have led us to propose the hypothesis that an interruption of the renin-angiotensin system (RAS) activity at a genetic level would prevent hypertension on a permanent basis. F$_1$ and F$_2$ generations of offspring from a retroviral vector, LNSV- and LNSV-AT$_1$R-AS–treated SHR, were generated, and various physiological parameters indicative of hypertension were studied and compared with those of their parents to investigate this hypothesis. Both F$_1$ and F$_2$ generations of LNSV-AT$_1$R-AS–treated SHR expressed a persistently lower blood pressure, decreased cardiac hypertrophy and fibrosis, decreased medial thickness, and normalization of renal artery excitation-contraction coupling, Ca$^{2+}$ current, and Ca$^{2+}$_i when compared with offspring derived from the LNSV-treated SHR. In fact, the magnitude of the prevention of these pathophysiological alterations was similar to that observed in the LNSV-AT$_1$R-AS–treated SHR parent. The prevention of cardiovascular pathophysiology and expression of normotensive phenotypes are, at least in part, a result of integration and subsequent transmission of AT$_1$R-AS from the SHR parents to offspring. These data demonstrate that a single intracardiac injection of LNSV-AT$_1$R-AS causes a permanent cardiovascular protection against hypertension as a result of a genomic integration and germ line transmission of the AT$_1$R-AS in the SHR offspring. The full text of this article is available at http://www.circresaha.org.

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Key Words: AT$_1$ receptor antisense | gene therapy | hypertension | SHR | antisense transmission to offspring

Hypertension is a complex disease that is associated with major economic and emotional burdens to society. It is also a significant risk factor for stroke, arteriosclerosis, heart failure, coronary artery disease, and progressive renal damage. Decades of investigation have established that a hyperactive renin-angiotensin system (RAS) is one of the many physiological events that becomes dysfunctional in hypertension. This fact is further supported by the observation that pharmacological interruption in the activity of the RAS has proved to be highly successful in the treatment and management of hypertension in a significant population of hypertensive patients. In spite of this success, the traditional pharmacological therapy targeted to inhibit specific components of the RAS suffers from many significant disadvantages. One is patient compliance. This is particularly important in view of the fact that chronic administration of drugs is almost always necessary for a persistent, long-term antihypertensive effect, and prolonged therapy with certain antihypertensive agents can lead to significant side effects such as coughing, angioedema, hypotension, renal dysfunction, and hyperkalemia. Additionally, although a traditional pharmacological strategy can be successful in the control and the management of high blood pressure (BP), its effectiveness in the prevention and/or reversal of other associated pathophysiological alterations such as tissue remodeling leading to end-organ damage remains to be proven. In fact, disproportionate reversal of some alterations, such as left ventricular hypertrophy and peripheral resistance, has proven to be unfavorable in the successful management of this disease.

In view of the success of pharmacological agents targeted toward the inhibition of the RAS, and the recent rapid advances in gene delivery, we decided to investigate whether antisense gene therapy could be a superior treatment for hypertension. We used a retrovirally mediated delivery system to administer AT$_1$ receptor antisense (AT$_1$R-AS) cDNA...
in vitro. These studies established that AT1R-AS cDNA could be incorporated into the genome and that the transcript could be expressed on a long-term basis. This expression was associated with a significant alteration of the AT1R-mediated cellular action of angiotensin II (Ang II) indicating that such an approach was feasible. Animal experiments were highly successful and demonstrated that intracardiac delivery of AT1R-AS in the neonatal spontaneously hypertensive rat (SHR) prevented the development of hypertension, renal, and cardiovascular pathophysiological changes on a long-term basis. The antihypertensive effect was associated with a robust long-term expression of AT1R-AS transcript. These studies led us to hypothesize that the interruption in the activity of the RAS during development by the AT1R-AS would attenuate hypertension on a permanent basis. The present study was designed to support or refute this hypothesis.

Materials and Methods
Preparation of Viral Particles Containing AT1R-AS
AT1R-AS was cloned in a retroviral vector containing long terminal repeats, genomic selection, and simian virus promoter (LNSV) as described previously. Media-containing viral particles from the PA317 cells were collected and concentrated to provide 1×10⁶ to 2×10⁹ cfu/mL as described previously. Viral particles that did not contain AT1R-AS (LNSV) were also prepared by the same protocol and used in control experiments.

Administration of LNSV- and LNSV-AT1R-AS-Containing Viral Particles in Rats
Five-day-old Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats were divided into two groups: virus-along control (LNSV) or virus-containing AT1R-AS (LNSV-AT1R-AS). Animals were injected with a bolus of 1×10⁶ to 10⁹ cfu/mL of viral particles in 10 μL physiological saline intracardially, weaned, and raised as described previously. Indirect BP was monitored throughout development. Two sets of parents of LNSV- or LNSV-AT1R-AS–treated WKY and SHR were bred at 100 days of age to generate F1 offspring. One hundred-day-old F1 generation was bred to generate F2 generation offspring. At day 120 of age, rats were cannulated, and direct BP was measured. The number of animals for WKY and SHR parents was 16; for F1 (n=12) and F2 (n=10) offspring, the number of animals was 22. Significantly different (P<0.02) from WKY rats. Significantly lower BP in LNSV-AT1R-AS–treated SHR parents and F1 and F2 offspring (P<0.005) compared with the LNSV-treated control SHR. The antihypertensive effect was associated with a robust long-term expression of AT1R-AS transcript. These studies led us to hypothesize that the interruption in the activity of the RAS during development by the AT1R-AS would attenuate hypertension on a permanent basis. The present study was designed to support or refute this hypothesis.

Pathophysiological Parameters
Heart weight-to-body weight ratio, cardiac fibrosis, and collagen volume in both endocardium and epicardium were determined by our previously published protocols. Animals were killed and perfused with fixative without applying additional pressure, according to a previously published protocol. Morphometric analysis of thoracic aorta to determine the wall thickness and media/lumen ratio was carried out by an established protocol.

Statistics
Results are expressed as mean±SE. Statistical significance was evaluated with repeated measures, ANOVA, and Student’s t test for unpaired data. Differences were considered significant at P<0.05. For vascular studies, all rings were normalized to tissue weights and cross-sectional area.

Results
Five-day-old WKY rats and SHR were administered 1×10⁶ cfu of LNSV (control virus) or LNSV-AT1R-AS (LNSV virus containing AT1R-AS cDNA) via an intracardiac injection. Animals were allowed to grow and were subjected to routine indirect BP monitoring. Two sets of LNSV-treated WKY and SHR were subjected to LNSV or LNSV-AT1R-AS treatment essentially as described previously. One hundred and twenty–day-old parents were bred to produce F1 generation of offspring. By 120 days, the average BP of the AT1R-AS–treated SHR offspring was significantly lower than that of the LNSV-treated SHR offspring (153±11 mm Hg, Figure 1). No significant effect on BP was observed between offspring of AT1R-AS– and LNSV-treated WKY rats. The LNSV-AT1R-AS–treated WKY rat group was not used further in our experimen-
The effect of losartan, an AT1 receptor–specific antagonist and well-established antihypertensive drug, on offspring derived from AT1R-AS–treated SHR was also studied. Treatment of control SHR by losartan resulted in a 29±6 mm Hg decrease in BP (Figure 3). A comparable decrease in BP was observed in LNSV-treated SHR. In contrast, no significant decrease in BP was observed in the LNSV-AT1R-AS–treated SHR. Similar to AT1R-AS–treated parents, their F1 and F2 offspring showed little lowering of BP by losartan whereas offspring from LNSV-treated parents experienced a 25 to 29 mm Hg decrease in BP (Figure 3). These observations confirm that the AT1R-AS treatment of parents produced antihypertensive effects in both parents and offspring through an AT1 receptor–mediated mechanism and that the antihypertensive effect is as effective as the AT1 receptor antagonist therapy. The conclusion that antisense gene therapy influences BP in the SHR by affecting the levels of AT1 receptors is demonstrated by comparing the cardiac AT1 receptors in the F1 generation of LNSV- and AT1R-AS–treated SHR. Total numbers of AT1 receptors (Bmax) in the ventricles of the F1 offspring derived from AT1R-AS–treated SHR were decreased by 36% compared with offspring from parents of LNSV-treated SHR (Table 1).

Tissue remodeling and associated ultrastructural changes in tissues relevant to cardiovascular functions such as heart, kidney, and arteries are major contributing factors in the morbidity and mortality associated with hypertension. For example, left ventricular hypertrophy, a compensatory response of the heart to an increase in peripheral vascular resistance is an important pathophysiological manifestation of hypertension. We determined whether AT1R-AS treatment influences cardiac pathophysiology, and, if so, could this effect be maintained in their offspring. Heart weights of untreated SHR were 68% higher than those of WKY rats (Table 1). AT1R-AS treatment significantly prevented this cardiac hypertrophy in parents. Similarly, heart weights of the F1 generation of AT1R-AS–treated SHR were 26% lower than the F1 generation of LNSV-treated SHR (Table 1). Cardiac hypertrophy was significantly prevented in the F2 generation of AT1R-AS–treated SHR as well. Multifocal areas of fibrosis in the myocardium are another characteristic of hypertension in this model. Figure 4 provides an example decrease in BP (Figure 3).
of sections taken from the left ventricular subendomyocardium of F2 offspring derived from LNSV- and AT1R-AS–treated SHR. Multiple areas of fibrosis were clearly evident in the offspring of AT1R-AS–treated animals (Figure 4c) or in the control WKY rat (Figure 4a). Collagen volume in both endocardium and epicardium, a measure of cardiac fibrosis, was 90% decreased in the offspring of LNSV-AT1R-AS SHR (Table 1), confirming the morphological detection of fibrosis.

Ultrastructural examination of the thoracic aorta of the offspring of LNSV-AT1R-AS–treated SHR parents revealed a significant decrease in the wall and medial thickness compared with offspring of LNSV-treated SHR parents (Table 1). The lumen area was increased in this group of SHR offspring. For example, wall thickness in the SHR and LNSV-treated SHR offspring of F2 generation was 36% to 90% greater than that of the WKY control. F2 offspring from LNSV-AT1R-AS–treated SHR demonstrated a 34% decrease in wall thickness, which was closer to values for the same measurement in WKY rats. Similarly, the media/lumen ratio was 42% lower in this generation of LNSV-AT1R-AS–treated SHR. These data clearly establish that AT1R-AS treatment prevents these vascular pathophysiological changes in this model of hypertension.

We examined the pathophysiological changes in the renal resistance arterioles and artery of the F1 and F2 offspring of AT1R-AS–treated SHR parents. The rationale was based on the fact that an increased vascular tone leading to an increased renal vascular resistance is an important underlying mechanism in the elevation of BP. Cellular mechanisms responsible for this include an enhanced contractile sensitivity to vasoactive agents, an impaired endothelial-dependent vasorelaxation, increased [Ca2+]i, by its transport across the vascular smooth muscle cell (VSMC) membrane, altered ion channel activity in VSMC, and smooth muscle cell hypertrophy and hyperplasia. We examined the effect of parental treatment of AT1R-AS of the SHR on the above pathophysiological parameters in the renal resistance arteriole and renal artery in the F1 and F2 offspring. Ultrastructural examination revealed that the thickness of the intima and media and the overall arterial morphological changes characteristic of hypertension were prevented in the SHR offspring of AT1R-AS–treated parents (Figure 4d through 4f).

Next, we examined the effects of AT1R-AS treatment on renal vascular reactivity. Our previous studies have shown that the SHR renal arteriole expresses an enhanced contractile response to KCl and phenylephrine. This enhancement, a result of a leftward shift of the concentration-response curve reflecting in the EC50 for KCl and phenylephrine, was attenuated in AT1R-AS–treated SHR. In the present study, the renal vascular response in offspring of both F1 and F2 generations of parents treated with LNSV-AT1R-AS was examined. In the parents, the vascular contractile responses to KCl and phenylephrine were shifted rightward as a result of an increase in EC50 values in both F1 and F2 generation of offspring from LNSV-AT1R-AS–treated SHR. Data for the F2 generation are presented in Table 2 as an example. As a result, the EC50 values for F1 and F2 generations from the LNSV-AT1R-AS–treated SHR parents were comparable to those in the WKY rat. In contrast to the result with KCl and phenylephrine when compared with WKY, the untreated and LNSV-treated SHR showed a shift to the right in the acetylcholine-induced vasorelaxation of precontracted renal arteriole as reflected by an increase in the EC50 as well as a decrease in the maximal effect. This effect was significantly improved in the offspring of parents treated with LNSV-AT1R-AS. For example, the EC50 response of F2 offspring of

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WKY Rat*</th>
<th>SHR*</th>
<th>LNSV-SHR</th>
<th>LNSV-AT1R-AS-SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight/body weight††</td>
<td>2.9±0.7</td>
<td>4.9±0.8</td>
<td>4.2±0.6</td>
<td>3.1±0.8</td>
</tr>
<tr>
<td>Bmax for AT1 receptor‡</td>
<td>46±5</td>
<td>131±8</td>
<td>130±8</td>
<td>83±6</td>
</tr>
<tr>
<td>Cardiac fibrosis§</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Rare</td>
</tr>
<tr>
<td>Collagen volume§, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocardium</td>
<td>4.0±0.02</td>
<td>NA</td>
<td>4.8±0.4</td>
<td>0.5±0.05</td>
</tr>
<tr>
<td>Epicardium</td>
<td>0.3±0.02</td>
<td>NA</td>
<td>7.8±1.4</td>
<td>0.5±0.05</td>
</tr>
<tr>
<td>Thoracic aorta§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wall thickness, mm2</td>
<td>0.11±0.04</td>
<td>0.25±0.03</td>
<td>0.21±0.008</td>
<td>0.14±0.005</td>
</tr>
<tr>
<td>Media area, mm2</td>
<td>0.61±0.04</td>
<td>1.30±0.09</td>
<td>1.52±0.008</td>
<td>0.82±0.010</td>
</tr>
<tr>
<td>Lumenal area, mm2</td>
<td>4.10±0.10</td>
<td>3.20±0.04</td>
<td>3.00±0.300</td>
<td>4.40±0.120</td>
</tr>
<tr>
<td>Media/lumen (ratio)</td>
<td>0.28±0.01</td>
<td>0.37±0.02</td>
<td>0.35±0.010</td>
<td>0.20±0.030</td>
</tr>
</tbody>
</table>

*Data taken from Martens et al18 and Gelband et al.23
†Data are mean±SE, n=12.
‡AT1 receptor Bmax was determined essentially as described previously.14,15 F1 generation offspring were used for Bmax determination.21,22
§Morphometric data were collected and analyzed as described previously from the F2 generation of offspring.24
¶Values are significantly different (P<0.01) from the LNSV-SHR and SHR.
Values are significantly different (P<0.05, n=3) from the LNSV-SHR.
NA indicates not analyzed.
LNSV-AT1R-AS–treated SHR was 77% lower when compared with the LNSV-treated SHR and was similar to that of the WKY rat. Similarly, the efficacy was 2.2-fold higher and comparable to the WKY rat (Table 2). These data demonstrate that endothelial dysfunction associated with hypertension is prevented by AT1R-AS gene therapy on a permanent basis.

We also studied L-type Ca\(^{2+}\) current in F1 and F2 generations from LNSV- and LNSV-AT1R-AS–treated SHR parents. The rationale for this experiment was based on our previous observations that demonstrated that L-type Ca\(^{2+}\) current is increased in VSMCs of the renal arterioles of the SHR.\(^{23}\) Ca\(^{2+}\) current was significantly decreased in both F1 and F2 generations of LNSV-AT1R-AS–treated SHR parents compared with that from the LNSV-treated SHR parent. The mean I-V relationship demonstrating this conclusion is presented in Figure 5A for the F2 generation of offspring. Differences in the peak Ca\(^{2+}\) current are shown in Table 2.

**TABLE 2. Pathophysiological Parameters of Renal Arteriole in Offspring of Parents Treated With LNSV-AT1R-AS**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WKY Rat*</th>
<th>SHR*</th>
<th>LNSV-SHR</th>
<th>LNSV-AT1R-AS-SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl constriction (EC(_{50}), nmol/L)*</td>
<td>35(\pm)2†</td>
<td>24(\pm)1</td>
<td>19(\pm)3</td>
<td>42(\pm)6†</td>
</tr>
<tr>
<td>Phenylephrine constriction (EC(_{50}), nmol/L)*</td>
<td>380(\pm)10†</td>
<td>202(\pm)13</td>
<td>138(\pm)7</td>
<td>483(\pm)10†</td>
</tr>
<tr>
<td>Acetylcholine vasorelaxation (EC(_{50}), nmol/L)*</td>
<td>30.7(\pm)5†</td>
<td>143(\pm)9</td>
<td>168(\pm)11</td>
<td>38(\pm)6†</td>
</tr>
<tr>
<td>Acetylcholine vasorelaxation (efficacy percent)*</td>
<td>100†</td>
<td>40(\pm)3</td>
<td>42(\pm)4</td>
<td>100†</td>
</tr>
<tr>
<td>Peak Ca(^{2+}) current ([\text{Ca}^{2+}]), pA/pF</td>
<td>1.1(\pm)0.01†</td>
<td>2.2(\pm)0.02</td>
<td>2.4(\pm)0.01</td>
<td>0.95(\pm)0.01</td>
</tr>
<tr>
<td>% Increase in ([\text{Ca}^{2+}]), %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>118(\pm)7†</td>
<td>228(\pm)13</td>
<td>240(\pm)11</td>
<td>122(\pm)8†</td>
</tr>
<tr>
<td>Ang II</td>
<td>192(\pm)6†</td>
<td>306(\pm)4</td>
<td>312(\pm)12</td>
<td>188(\pm)11†</td>
</tr>
</tbody>
</table>

Data are mean\(\pm\)SE (n=24) and taken from F2 generation of offspring.

*Data taken from Martens et al\(^{18}\) and Gelband et al\(^{23}\) for comparison.

†Values are significantly different (P<0.01) from LNSV-SHR and SHR.
Finally, we investigated the effects of KCl and Ang II on [Ca^{2+}] in renal arteriolar VSMCs. Our previous studies have established that KCl- and Ang II–induced [Ca^{2+}], were significantly elevated in the SHR compared with the WKY rat. Data in Figure 5B show that KCl- or Ang II–induced [Ca^{2+}] in F2 offspring of LNSV-AT1R-AS–treated SHR parents were significantly different (P<0.01) from LNSV-AT1R-AS group of offspring (n=270 cells from 6 animals).

Figure 5. A, Mean I-V relationship for L-type Ca^{2+} current in offspring of LNSV-AT1R-AS–treated SHR parents. The Ca^{2+} current density of LNSV- or LNSV-AT1R-AS–treated SHR offspring was determined in VSMCs of renal resistance arteriole essentially as described previously. B, KCl- and Ang II–mediated changes in [Ca^{2+}]i in F2 offspring of LNSV-AT1R-AS–treated SHR parents. Experimental protocols for LNSV- and LNSV-AT1R-AS–treated SHR were essentially as described previously. *Significantly different (P<0.01) from LNSV-AT1R-AS group of offspring.

Discussion

The observations presented in this study demonstrate that a single intracardial administration of a retroviral vector containing AT1R-AS causes a permanent protection against hypertension in this animal model of human primary hypertension. Thus, it suggests that an antisense strategy to inhibit the RAS at a genetic level is a conceptually novel approach for the prevention of hypertension. The reduction in BP is well-established that ACE gene polymorphism cosegregates with hypertension, and that mutations at key places about the efficiency of this transduction, it must be high enough to influence the expression of antihypertensive phenotypes, an end point that is of ultimate relevance to hypertension. The possibility that lack of a blood-gonadal barrier and the presence of significant numbers of undifferentiated germ cells in the neonatal rat cannot be ruled out. Thus, a critical age of the rat at which the viral administration was carried out may be the key for such a high efficiency of transduction in the offspring.

In spite of our evidence in favor of AT1R-AS transmission, other possibilities to explain this prolonged antihypertensive effect should not be ruled out at the present time. For example, studies have shown that parental environment is critical in the development of hypertension. Thus, it is quite possible that the exposure of an antihypertensive environment by the AT1R-AS treatment of parents induces normotensive phenotype in the offspring. Cross studies with the SHR would support this review.

In addition, the possibility that the AT1R-AS expression at a critical stage of SHR development may irreversibly prevent the parents and offspring from developing hypertension. This would be consistent with previous suggestions. Finally, it is also quite possible that a combination of these mechanisms may ultimately be responsible for such a dramatic protection against hypertension.

The most important question that arises from the study concerns the mechanism of by which normotensive phenotypes are transmitted from parents to offspring. Our data in Figure 2 support the notion that the AT1R-AS is integrated into the parental genome and is transmitted to the offspring. The proposed germ-line transmission of the AT1R-AS is consistent with previous reports demonstrating the integration of retroviral vector and its germ-line transmission in other systems. However, this study is unique because it shows that the transmission and accompanied expression of the AT1R-AS is associated with profound antihypertensive physiological changes in the offspring. Although we know little about the efficiency of this transduction, it must be high enough to influence the expression of antihypertensive phenotypes, an end point that is of ultimate relevance to hypertension. The possibility that lack of a blood-gonadal barrier and the presence of significant numbers of undifferentiated germ cells in the neonatal rat cannot be ruled out. Thus, a critical age of the rat at which the viral administration was carried out may be the key for such a high efficiency of transduction in the offspring.
sion, our observation provides an initial step forward toward the use of gene therapy for a permanent benefit to the cardiovascular system in the control of hypertension.

Acknowledgments
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