Permanent Cardiovascular Protection From Hypertension by the AT₁ 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 (AT₁, 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. F₁ and F₂ generations of offspring from a retroviral vector, LNSV- and LNSV-AT₁, 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 F₁ and F₂ generations of LNSV-AT₁, 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⁺⁺ current, and [Ca²⁺]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-AT₁, 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 AT₁, R-AS from the SHR parents to offspring. These data demonstrate that a single intracardiac injection of LNSV-AT₁, R-AS causes a permanent cardiovascular protection against hypertension as a result of a genomic integration and germ line transmission of the AT₁, R-AS in the SHR offspring. The full text of this article is available at http://www.circresaha.org. (Circ Res. 1999;85:e44–e50.)

Key Words: AT₁ 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 in stroke, arteriosclerosis, heart failure, coronary artery disease, and progressive renal damage.4,5 Decades of investigation have established that a hyperactive renin-angiotensin system (RAS) is one of the many physiological events that becomes dysfunctional in hypertension.2–5 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.6,7 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.8–10 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.11 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.12,13

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 AT₁ receptor antisense (AT₁, R-AS) cDNA...
in vitro. These studies established that AT1-R-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 AT1-R-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 AT1-R-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 AT1-R-AS transcript. These studies led us to hypothesize that the interruption in the activity of the RAS during development by the AT1-R-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 AT1-R-AS
AT1-R-AS was cloned in a retroviral vector containing long terminal repeats, the gibbon virus promoter (LNSV) as described previously. Media-containing viral particles from the PA317 cells were collected and concentrated to provide 1×10^9 to 2×10^10 cfu/mL as described previously. Viral particles that did not contain AT1-R-AS (LNSV) were also prepared by the same protocol and used in control experiments.

Administration of LNSV- and LNSV-AT1-R-AS-Containing Viral Particles in Rats
Five-day-old Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats were divided into two groups, virus-alone control (LNSV) or virus-containing AT1-R-AS (LNSV-AT1-R-AS). Animals were injected with a bolus of 1×10^9 to 3×10^9 cfu 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-AT1-R-AS–treated WKY and SHR were bred at 100 days of age to generate F1 offspring. Similar to their parents, they were weaned, raised, and monitored for BP indirectly by the tail-cuff method. At 100 days of age, two sets of F1 offspring of LNSV- and LNSV-AT1-R-AS–treated WKY and SHR were bred to generate F2 offspring. One hundred-day-old parents and F1 and F2 offspring were used for all biochemical and physiological experiments.

Biochemical Experiments
Binding of 125I-Sar1-Ile8-Ang II to membrane AT1 receptors was carried out as described elsewhere. Computer-assisted Scatchard analysis was done to determine the Bmax and Kd values. Polynucleotide chain reaction (PCR) followed by a Southern analysis was carried out to determine the genomic integration of the AT1-R-AS essentially as described elsewhere. The expression of AT1-R-AS transcript in various Ang II target tissues was carried out by a semiquantitative reverse transcriptase (RT)-PCR method as we described previously.

Physiological Protocols
Indirect BP was monitored in nonanesthetized animals by a standard tail-cuff method. Direct BP was determined in free-moving animals as previously described. For vascular smooth muscle studies, 3-mm-long segments of rat renal resistance arteries were used. Contractile responses to KCl, phenylephrine, and acetylcholine were evaluated as described previously. [Ca2+]i, in dissociated renal resistance arterial cells in response to KCl and Ang II was measured exactly as we described previously. Current recordings were carried out by voltage-clamping single cells.

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 1×10^9 cfu of LNSV (control virus) or LNSV-AT1-R-AS (LNSV virus containing AT1-R-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 rats (mean BP 129±6.0 mm Hg) and SHR (mean BP 198±18 mm Hg) and LNSV-AT1-R-AS–treated WKY and SHR (mean BP 120±6.0 and 153±9 mm Hg, respectively) were used as parents for breeding and an F1 generation of rats was produced. Offspring from LNSV-AT1-R-AS–treated SHR expressed significantly lower BP as early as 80 days of age compared with their LNSV-treated SHR control. By 120 days, the average BP of the AT1-R-AS–treated SHR offspring was of 45±9 mm Hg lower than that observed in the LNSV-treated SHR offspring (150±10 versus 185±11 mm Hg, Figure 1). No significant effect on BP was observed between offspring of AT1-R-AS– and LNSV-treated WKY rats. The LNSV-AT1-R-AS–treated WKY rat group was not used further in our exper-
The effect of losartan, an AT$_1$ receptor–specific antagonist and well-established antihypertensive drug, on offspring derived from AT$_1$R-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-AT$_1$R-AS–treated SHR. Similar to AT$_1$R-AS–treated parents, their F$_1$ and F$_2$ 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 AT$_1$R-AS treatment of parents produced antihypertensive effects in both parents and offspring through an AT$_1$ receptor–mediated mechanism and that the antihypertensive effect is as effective as the AT$_1$ receptor antagonist therapy. The conclusion that antisense gene therapy influences BP in the SHR by affecting the levels of AT$_1$ receptors is demonstrated by comparing the cardiac AT$_1$ receptors in the F$_1$ generation of LNSV- and AT$_1$R-AS–treated SHR. Total numbers of AT$_1$ receptors (B$_{max}$) in the ventricles of the F$_1$ offspring derived from AT$_1$R-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 AT$_1$R-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). AT$_1$R-AS treatment significantly prevented this cardiac hypertrophy in parents. Similarly, heart weights of the F$_1$ generation of AT$_1$R-AS–treated SHR were 26% lower than the F$_1$ generation of LNSV-treated SHR (Table 1). Cardiac hypertrophy was significantly prevented in the F$_2$ generation of AT$_1$R-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 of a fibrotic heart from an AT$_1$R-AS–treated SHR.
of sections taken from the left ventricular subendomyocardium of F_2 offspring derived from LNSV- and AT_1 R-AS–treated SHR. Multiple areas of fibrosis were clearly evident in the offspring of LNSV-treated SHR (Figure 4b) but were rarely observed in the offspring of AT_1 R-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-AT_1 R-AS SHR (Table 1), confirming the morphological detection of fibrosis.

Ultrastructural examination of the thoracic aorta of the offspring of LNSV-AT_1 R-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 F_2 generation was 36% to 90% greater than that of the WKY control. F_2 offspring from LNSV-AT_1 R-AS–treated parents revealed a significant decrease in the wall and medial thickness compared with offspring of LNSV-SHR and SHR.

Table 1. Cardiac Pathophysiological Parameters in Offspring of Parents Treated With LNSV-AT_1 R-AS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WKY Rat*</th>
<th>SHR*</th>
<th>LNSV-SHR</th>
<th>LNSV-AT_1 R-AS-SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight/body weight†, mg/g</td>
<td>2.9±7</td>
<td>4.9±8</td>
<td>4.2±0</td>
<td>3.1±0.8</td>
</tr>
<tr>
<td>B_max for AT_1 receptor‡, fmol/mg protein</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>Medial 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 al^18 and Gelband et al^23
†Data are mean±SE, n=12.
‡AT_1 receptor B_max was determined essentially as described previously. 14,15 F_1 generation offspring were used for 125I-Sar¹ Ile⁸–Ang II binding experiments. Data are mean±SE (n=3).
§Morphometric data were collected and analyzed as described previously from the F_2 generation of offspring. 24 Data are mean±SE (n=3).

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.

Next, we examined the effects of AT_1 R-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 EC_50 for KCl and phenylephrine, was attenuated in AT_1 R-AS–treated SHR. In the present study, the renal vascular response in offspring of both F_1 and F_2 generations of parents treated with LNSV-AT_1 R-AS was examined. In the parents, the vascular contractile responses to KCl and phenylephrine were shifted rightward as a result of an increase in EC_50 values in both F_1 and F_2 generation of offspring from LNSV-AT_1 R-AS–treated SHR. Data for the F_2 generation are presented in Table 2 as an example. As a result, the EC_50 values for F_1 and F_2 generations from the LNSV-AT_1 R-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 EC_50 as well as a decrease in the maximal effect. This effect was significantly improved in the offspring of parents treated with LNSV-AT_1 R-AS. For example, the EC_50 response of F_2 offspring of...
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 F\(_1\) and F\(_2\) 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 F\(_1\) and F\(_2\) 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 F\(_2\) generation of offspring. Differences in the peak Ca\(^{2+}\) current are shown in Table 2.

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Morphological changes in myocardium and renal artery in F\(_2\) offspring of LNSV-AT1R-AS–treated SHR parents. Hearts of F\(_2\) generation offspring of WKY (a), LNSV-treated SHR (b), and LNSV-AT1R-AS–treated SHR (c) parents were used to determine fibrosis essentially as described elsewhere.\(^{18}\) Bar=12 mm. Arrows show multifocal areas of fibrosis. F\(_2\) rats were killed, and renal arteries were dissected from the main aorta and fixed in 3% paraformaldehyde.\(^{24}\) Sections from 3 mm distal from the aorta were compared in WKY (d), LNSV-treated SHR (e), and LNSV-AT1R-AS–treated SHR parents (f). Bar=1 mm.

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

Data are mean±SE (n=24) and taken from F\(_2\) 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.
This Article Has Been Retracted
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

This research was supported by grants from the National Institutes of Health (HL56921 and HL52189). Drs Phyllis Reaves and Hong Yang are postdoctoral fellows of the American Heart Association, Florida Affiliate. We thank Dr Craig Tishler, University of Florida, for his intellectual input throughout this study and his critical review of the manuscript. Technical support of Ling Liu and Rebecca Kochera and editorial help of Marya Fancey are gratefully acknowledged.

References

Permanent Cardiovascular Protection From Hypertension by the AT₁ 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 and Mohan K. Raizada

Circ Res. 1999;85:e44-e50
doi: 10.1161/01.RES.85.10.e44

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/10/e44

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/