Angiotensin I–Converting Enzyme Antisense Gene Therapy Causes Permanent Antihypertensive Effects in the SHR

Hongwei Wang, Phyllis Y. Reaves, Monica L. Gardon, Kimberley Keene, Drew S. Goldberg, Craig H. Gelband, Michael J. Katovich, Mohan K. Raizada

Abstract—The renin-angiotensin system plays a critical role in the control of blood pressure (BP), and its hyperactivity is associated with the development and maintenance of hypertension. Although traditional pharmacological therapies targeted toward the inhibition of the renin-angiotensin system are effective in the control of this disease, they pose significant limitations. We used an antisense gene delivery strategy to circumvent these limitations and established that a single intracardiac administration of angiotensin type 1 receptor antisense (AT1 R-AS) causes permanent prevention of hypertension in the spontaneously hypertensive rat (SHR), an animal model of primary human hypertension. Our objectives in this study were 2-fold: to determine (1) whether the targeting of angiotensin I–converting enzyme (ACE) mRNA by a similar antisense strategy would prevent the SHR from developing hypertension and (2) whether the antihypertensive phenotype is transmitted to the offspring from the antisense-treated parents. Administration of a retroviral vector containing ACE antisense (LNSV-ACE-AS) caused a modest yet significant attenuation of high BP (≈15 ± 2 mm Hg) exclusively in the SHR. This was associated with a complete prevention of cardiac and renovascular pathophysiological alterations that are characteristic of hypertension. Like their parents, the F1 generation offspring of the LNSV-ACE-AS–treated SHR expressed lower BP, decreased cardiac hypertrophy, and normalization of renal arterial excitation-coupling compared with offspring derived from the LNSV-ACE-tS (truncated sense)–treated SHR. In addition, the endothelial dysfunction commonly observed in the SHR renal arterioles was significantly prevented in both parents and offspring of the LNSV-ACE-AS–treated SHR. Polymerase chain reaction followed by Southern analysis revealed that the ACE-AS was integrated into the SHR genome and transmitted to the offspring. These observations suggest that transmission of ACE-AS by retroviral vector may be responsible for the transference of normotensive phenotypes in the SHR offspring. (Hypertension. 2000;35[part 2]:202-208.)

Key Words: SHR ■ viral delivery ■ hypertension ■ cardiac hypertrophy ■ renovascular responsiveness

Hypertension is a complex disease that manifests as chronically high blood pressure (BP). It is a major risk factor in many cardiovascular pathophysiological states, including atherosclerosis, stroke, heart failure, coronary artery disease, and progressive renal damage.1–3 Overwhelming evidence has established that a dysfunctional renin-angiotensin system (RAS) is one of the many physiological alterations that contribute to the development and establishment of hypertension.4–6 This conclusion is based on the fact that the traditional pharmacological therapies targeted to inhibit the activity of the RAS are a highly successful strategy for the treatment and management of this disease in a significant population of hypertensive patients.7–10 Despite their success, traditional pharmacological agents, such as angiotensin I–converting enzyme inhibitors (ACE-I) and the AT1 receptor (AT1 R) antagonists, have major limitations that include compliance, side effects, and relatively short duration of antihypertensive effects.11,12 As a result of these limitations, the current therapeutic strategy has reached a plateau, and conceptually innovative and novel approaches must be explored to advance the field of hypertension therapies.

Our research group has begun to use an antisense gene therapy approach to determine whether targeting of the AT1 receptor at a genetic level is a step toward long-term control of hypertension. These studies have revealed that a single intracardiac administration of retroviral vector containing AT1 R-antisense (AS) results in a long-term prevention of high BP in the spontaneously hypertensive rat (SHR).13,14 This is associated with the prevention of renovascular and cardiac pathophysiological changes that are characteristic of hypertension.15,16 In view of these observations, we set out to investigate the following objectives: (1) We wanted to determine whether the targeting of another component of the RAS at a genetic level with a similar antisense strategy would produce long-lasting antihypertensive effects as seen with the ATR-AS. This, we argued, would be an essential prerequisite to prove the conceptual validity of an antisense gene therapy

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approach. (2) Would the normotensive phenotypes be passed on to offspring from the antisense-treated parents? If so, what would be the possible mechanism? The observations presented here establish that the delivery of ACE-AS by retroviral vector in neonatal rats results in a modest yet long-term decrease of high BP in the SHR. The modest decrease, however, was accompanied by a complete prevention of cardiac and renovascular pathophysiology. These corrected phenotypes are transferred from the parents to their offspring.

Methods

Construction of LNSV Containing ACE-tS and ACE-AS

A retroviral vector, LNSV, was used to deliver ACE-AS and ACE-tS into rats. The sense orientation of the ACE cDNA is only ~1.0 kb and not full length. As a result, it would not generate an active ACE. Thus, we called the sense orientation truncated sense (tS), which serves as an excellent control for the antisense orientation of ACE. Rat ACE cDNA was generated by reverse transcription–polymerase chain reaction (RT-PCR) with the use of ACE specific primers (sense: 5'-GGCCTGACACCAATCTACGAGGAGGAAA-3'; antisense: 5'-ATGTCGACCCCGTGCATTCTAAT-3') that corresponded to nt 254 to nt 1181 as previously described. A map of relevant LNSV-ACE-tS/AS with various restriction sites is represented in Figure 1. These restriction enzymes were used to characterize the recombinants. For example, a SacI digestion of LNSV-ACE-tS provided predicted bands corresponding to ~1.2, 2.9, and 3.1 kb. Bands of ~0.9, 3.0, and 3.2 kb were specific for a similar digestion of LNSV-ACE-AS. EcoRI digestion provided 3 predicted bands of ~1.6, 2.3, and 3.2 kb for LNSV-ACE-tS and 3 bands of ~1.6, 1.7, and 3.8 kb for LNSV-ACE-AS. Final characterization of these bands and their sense and antisense orientations was carried out through sequence analysis.

Preparation of Culture Media Containing LNSV-ACE-tS and LNSV-ACE-AS

PA317 cells in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum were used for transfection and preparation of viral particles as described previously.14–17

Physiological Measurements

Five-day-old Wistar-Kyoto rats (WKY) and SHR were divided into control (vehicle), viral control (LNSV-ACE-tS), and experimental (LNSV-ACE-AS) groups. The animals were lightly anesthetized with methoxyflurane (Metofane, Mallinckrodt Veterinary, Inc). They were injected with 10^5 μL, via cardiac route, of either physiological saline (control) or 5×10^5 colony-forming units of viral particles containing LNSV-ACE-tS (viral control) or LNSV-ACE-AS (experimental) as described previously.14–17 Polybrene (Sigma Chemical Co) at a concentration of 7 mg/mL was added to the viral medium before injection.

Indirect systolic BPs were measured at regular intervals in all animals by the tail-cuff method as described previously. Carotid and jugular cannulations were carried out for the measurement of direct BPs in free-moving, nonrestrained animals essentially as described previously.

WKY and SHR parents who were treated with either LNSV-ACE-tS or LNSV-ACE-AS at 5 days of age were used for breeding. A pair of 120-day-old LNSV-ACE-tS males were bred with a pair of LNSV-ACE-AS females of comparable age to generate LNSV-ACE-tS offspring. Similarly, LNSV-ACE-AS males were mated with the LNSV-ACE-AS–treated females to generate LNSV-ACE-AS F1 offspring.

Animals were euthanized, and the hearts and kidneys were excised in physiological saline. Ventricular hypertrophy and renal arteriolar reactivity was performed as previously described.

Statistical Analysis

All results are expressed as mean±SEM. Indirect BP measurements were performed on 6 to 12 animals per group (unless stated otherwise) and analyzed by repeated-measures ANOVA. Direct mean arterial pressure (MAP) was analyzed by 2-way ANOVA. Vascular reactivity was analyzed by constructing concentration-response relationships for each experiment. EC50 values were generated for each treatment, and statistical analysis was performed with ANOVA and Student’s t test. Values were considered significant at P<0.05.

Results

Effect of ACE-AS on BP

The effect on indirect BP of LNSV-ACE-AS administration to 5-day-old WKY and SHR was measured as a function of age. A truncated ACE sense (LNSV-ACE-tS) has been used as a control for the LNSV-ACE-AS. No significant difference in the BP was observed between LNSV-ACE-tS and LNSV-ACE-AS–treated WKY rats at any of the times evaluated (Figure 2). BP in saline-treated controls of WKY and SHR parents who were treated with either LNSV-ACE-tS or LNSV-ACE-AS at 5 days of age were used for breeding. A pair of 120-day-old LNSV-ACE-tS males were bred with a pair of LNSV-ACE-AS females of comparable age to generate LNSV-ACE-tS offspring. Similarly, LNSV-ACE-AS males were mated with the LNSV-ACE-AS–treated females to generate LNSV-ACE-AS F1 offspring.
ACE-tS–treated SHR began to express significantly higher BP by 63 days of age compared with LNSV-ACE-tS–treated WKY.

In contrast to WKY rats, LNSV-ACE-AS treatment resulted in a significant lowering of BP in the SHR by 63 days of age. An average decrease of 17±2 mm Hg BP by 92 days was observed in the LNSV-ACE-AS–treated SHR group compared with the LNSV-ACE-tS–treated SHR (Figure 2). At 100 days, MAP in both groups of WKY and SHR was measured to confirm this modest yet significant decrease in BP response of LNSV-ACE-AS exclusively in the SHR. The MAP in the LNSV-ACE-AS–treated SHR was significantly lower than the MAP of LNSV-ACE-tS–treated SHR. The effect of captopril on MAP was measured to determine whether this ACE inhibitor would further lower the BP in ACE-AS–treated rats in an attempt to confirm the exclusive antihypertensive effect in the SHR. As in the WKY rat, captopril treatment exhibited no significant lowering of MAP in the LNSV-ACE-AS–treated SHR (data not shown).

Plasma Angiotensins and Bradykinins in LNSV-ACE-AS–Treated Parents and Offspring

<table>
<thead>
<tr>
<th></th>
<th>Angiotensin I, pmol/L</th>
<th>Angiotensin II, pmol/L</th>
<th>BK (1–9), fmol/L</th>
<th>BK (1–7), fmol/L</th>
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<tr>
<td>Parents</td>
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<tr>
<td>WKY-tS</td>
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<td>8±3</td>
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<td>0.4±0.04</td>
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<tr>
<td>SHR-AS</td>
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<td>4±2</td>
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<tr>
<td>F1 offspring</td>
<td></td>
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<tr>
<td>WKY-tS</td>
<td>15±4</td>
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<td>0.1±0.01</td>
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<tr>
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<td>7±2</td>
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Figure 3. Effect of LNSV-ACE-AS on direct mean BP in WKY and SHR parents and offspring. Five-day-old WKY and SHR were treated with LNSV-ACE-tS or LNSV-ACE-AS as described in Figure 2. The 120-day-old parents were bred to produce F1 generation of offspring. Subsequently, 100-day-old F1 rats and parents were cannulated, and direct MAP was measured. Numbers of animals for WKY and SHR parents and offspring groups were 8 and 12, respectively. *P<0.001 vs WKY-ACE-tS; ▲, P<0.003 vs SHR-ACE-tS.

ACE inhibitors are known to reduce renin-angiotensin activity, which may lead to a decrease in aldosterone production and increased potassium excretion. A single dose of captopril treatment exhibited an average of 25±5 mm Hg lower MAP than their ACE-tS controls (Figure 3).

Effect of LNSV-ACE-AS on Cardiac Pathology and Renovascular Reactivity

Previous studies have established that the AT1 R-AS gene therapy prevents the development of cardiac pathophysiology in the SHR, including ventricular hypertrophy and myocardial and perivascular fibrosis. In view of this, coupled with our observation of only modest effects on BP, our first objective was to determine whether the genetic targeting of ACE would also result in a long-term prevention and, more importantly, whether this prevention was maintained in the F1 offspring. Heart weights of LNSV-ACE-tS–treated SHR parents and F1 offspring were 25% to 30% higher than those of the WKY (Figure 4). LNSV-ACE-AS treatment of parents resulted in a significant prevention of this cardiac hypertrophy in both parents and F1 offspring. The attenuation of hypertrophy was associated with a significant prevention of myocardial fibrosis in both parents and F1 offspring.

Alterations in the vascular contractile responses are known to exist in the SHR, and our studies have shown that the AT1 R-AS gene therapy prevents these alterations in the renal artery. Because an increase in vascular tone leading to an increased renovascular resistance is an important underlying

Figure 4. Cardiac hypertrophy in the LNSV-ACE-AS–treated WKY and SHR parents and offspring. Experimental conditions were essentially as we described previously. Four WKY and 8 SHR were used in each group. *P<0.003 vs WKY-ACE-tS; ▲, P<0.003 vs SHR-ACE-tS.
mechanism in hypertension, we investigated the possibility that ACE-AS treatment of the SHR parents would prevent this alteration in vascular tone in the offspring. Enhanced contractile responses to both phenylephrine and KCl were observed in the LNSV-ACE-tS SHR compared with the WKY rat as a result of a leftward shift in the dose-response relationship (Figures 5 and 6). ACE-AS treatment resulted in a rightward shift in the phenylephrine and KCl dose responses in the SHR such that the EC₅₀s were not different from those of the WKY rat (Figures 5 and 6). The contractile responses to phenylephrine and KCl of renal arteries from the F₁ offspring of LNSV-ACE-AS–treated SHR were similar to those observed in the LNSV-ACE-AS SHR parents exhibiting a rightward shift in the dose-response relationship and a decrease in the EC₅₀, associated with this change (Figures 5 and 6). These data demonstrate that alterations in both the receptor- and voltage-mediated contractile responses were prevented by ACE-AS treatment of the SHR, and this prevention was maintained in the offspring.

Finally, an impaired endothelium-dependent relaxation of precontracted renal arteries was observed in the LNSV-ACE-tS–treated SHR as a result of a 62% decrease in the maximal responsiveness compared with the WKY control. This decrease was prevented in the LNSV-ACE-AS–treated SHR such that the maximal responses in this group of rats were found to be similar to those of LNSV-ACE-tS–treated WKY. A similar correction of endothelial dysfunction in response to acetylcholine was maintained in the offspring of LNSV-ACE-AS–treated SHR (Figure 7). Thus, our data demonstrate a complete prevention of pathophysiological alterations, even when the BP responses are modest, by ACE-AS treatment.

Integration of ACE-AS
PCR followed by Southern analysis was carried out to determine whether the above-observed long-term antihypertensive responses that are transferred from parents to offspring are a result of integration of ACE-AS into the genome of parents and its subsequent transmission into the offspring. Figure 8 shows that retroviral vector containing ACE-AS was integrated into the genome of various angiotensin target tissues of parents who were injected with the LNSV-ACE-AS viral particles at 5 days of age. A similar pattern of integration was also observed in the offspring generated from these parents. This was associated with the expression of ACE-AS transcript in both parents and offspring (Figure 9).
Discussion

The most significant findings of this study are that (1) a single administration of the retroviral vector containing ACE-AS results in a long-term, modest yet significant lowering of high BP (nevertheless, a complete prevention of cardiac and renovascular pathophysiology was observed), and (2) the normotensive phenotype produced by ACE-AS delivery in parents is transmitted to offspring.

Delivery of ACE-AS and not ACE-tS causes a long-term modest decrease in the high BP exclusively in the SHR, an animal model of primary hypertension. This was associated with the prevention of cardiac and renovascular pathophysiology was observed, and (2) the normotensive phenotype produced by ACE-AS delivery in parents is transmitted to offspring.

Despite this important similarity, there appears to be a major difference between the AT,R-AS and ACE-AS approaches. ACE-AS treatment produced only a modest decrease in high BP (\(\sim 15 \text{ mm Hg}\)) compared with AT,R-AS gene therapy, in which the decrease was more pronounced (\(\sim 30 \text{ to } 40 \text{ mm Hg}\)). Despite a modest BP response, there was a complete prevention of cardiac and renovascular pathophysiology by the ACE-AS treatment (Figure 10). This observation supports clinical trial data indicating that low subpressure doses of ACE-I are able to induce beneficial effects in remodeling and pathophysiology of many cardiovascular system–relevant tissues.18–21 However, ACE-AS therapy could accomplish this by a single administration of the vector with long-term effects, whereas the traditional ACE-I therapy requires continuous treatment. The fact that ACE-AS can produce complete prevention of pathophysiology could provide us with an important experimental system to dissect out an age-old relationship between high BP and end-organ damage and other pathophysiological changes associated with hypertension. Thus, the model can provide valuable confirmation on the role of tissue versus endocrine RAS in the control of hypertension. Although the precise mechanism of this diversity between the control of high BP and the pathophysiological changes remains to be elucidated, it is
tempting to suggest that ACE-AS targets primarily tissue ACE and RAS and thus affects remodeling of cardiovascular system–relevant tissues more effectively. This view is supported, in part, by our observation indicating that circulating levels of Ang I, Ang II, and bradykinin are not altered by ACE-AS treatment.

A unique feature of this study is that it establishes that the normal phenotypes of the BP control system are transmitted from parents to offspring by ACE-AS treatment. The mechanism of such a profound transmission remains to be elucidated, although our evidence supports the notion that the antihypertensive trait transference could be a result of transmission of ACE-AS cDNA from the parents to offspring. Data in Figure 8 indicating a genomic integration of ACE-AS in various tissues of the SHR parents and offspring support this view. The integration is associated with a robust expression of ACE-AS in both parents and offspring. Thus, the observations clearly indicate that the transduction efficiency of the ACE-AS in Ang II target tissues must be high enough to influence the expression of pathophysiology of hypertension and thus is physiologically relevant. Lack of a blood-gonadal barrier and the presence of significant numbers of undifferentiated germ cells in the neonatal rat at the time of viral delivery could account for such a high efficiency of transduction.

The observation of a germ-line transmission of the ACE-AS from parents to offspring is consistent with previous observations clearly indicate that the transduction efficiency of the ACE-AS in both parents and offspring. Thus, the observation of a germ-line transmission of the ACE-AS in Ang II target tissues must be high enough to influence the expression of pathophysiology of hypertension and thus is physiologically relevant. Lack of a blood-gonadal barrier and the presence of significant numbers of undifferentiated germ cells in the neonatal rat at the time of viral delivery could account for such a high efficiency of transduction.

The observation of a germ-line transmission of the ACE-AS from parents to offspring is consistent with previous reports demonstrating the integration of retroviral vector and its germ-line transmission in other systems. However, this study is unique in 2 ways: first, it shows the transmission in a mammalian system; and second, the transmission is accompanied by profound physiological changes. Further linkage studies must be conducted to prove this germ-line transmission conclusively.

Finally, it is relevant to address whether the ACE-AS gene therapy is a significant advance over traditional hypertension therapies. On the basis of our data, the answer has to be yes. In the SHR, an animal model for primary hypertension, a single injection of ACE-AS offers the possibility of a modest but permanent reduction in BP and complete cardiovascular protection, which is transmitted to offspring. This, coupled with the fact that ACE polymorphism cosegregates with hypertension and high BP, indicates a new and exciting dimension in ACE-AS therapy. One caveat with the use of a retroviral vector in human gene therapy is that the permanent nature of the antihypertensive effect may not be appropriate in situations in which the therapeutic regimen must be interrupted because of adverse effects, pregnancy, etc. Therefore, a regulated expression system must be developed in which exogenous agents could control the expression of ACE-AS on demand and, in turn, regulate its therapeutic potential. Studies are currently under way to develop such a regulatable system. In conclusion, these data provide evidence for a possible germ-line transmission of ACE-AS to produce permanent antihypertensive action in the parents and offspring of the SHR.

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