Inability to Induce Hypertension in Normotensive Rat Expressing AT$_1$ Receptor Antisense

Alok S. Pachori, Hongwei Wang, Craig H. Gelband, Carlos M. Ferrario, Michael J. Katovich, Mohan K. Raizada

Abstract—Our previous studies have shown that neonatal delivery of angiotensin type 1 receptor antisense (AT$_1$R-AS) in a retroviral vector prevents spontaneously hypertensive rats from developing hypertension for life but has no effect on blood pressure (BP) in normotensive animals. Based on these results, we hypothesized that AT$_1$R-AS transduction in normotensive rats would protect them from developing experimental hypertension. The present study was designed to evaluate this hypothesis. A single intracardiac administration of AT$_1$R-AS by a retroviral-mediated delivery system (LNSV-AT$_1$R-AS) in 5-day-old normotensive Sprague-Dawley rats resulted in long-term expression of the AT$_1$R-AS without an effect on basal BP. However, angiotensin II (Ang II)–induced BP, dipsogenic responses, and renovascular contractility were significantly attenuated in the LNSV–AT$_1$R-AS–treated rats. Chronic infusion of low-dose Ang II (55 ng·kg$^{-1}$·min$^{-1}$) in LNSV-alone–treated rats caused a modest increase in BP, profound increase in cardiac hypertrophy, and increased vascular contractility. In contrast, the LNSV-AT$_1$R-AS–treated rats were protected from developing these changes after Ang II infusion. These data establish that LNSV-AT$_1$R-AS pretreatment protects healthy rats from developing Ang II–dependent hypertension. (Circ Res. 2000;86:1167-1172.)

Key Words: angiotensin II • gene therapy • renin-angiotensin system • angiotensin type 1 receptor • hypertension

The renin-angiotensin system (RAS) plays an important role in human primary hypertension. Inhibition of angiotensin II (Ang II) formation or interaction with the angiotensin type 1 receptor (AT$_1$R) is important in controlling hypertension.1–3 Despite great success with traditional therapy, the cure for hypertension does not seem to be forthcoming. This may be because of many inherent disadvantages with treatment approach, including compliance, side effects, and lack of complete reversal of pathophysiological aspects of the disease.4 Previously, we explored the possibilities of genetic intervention in the expression of the RAS to control hypertension and circumvent these issues. This has proven to be highly successful at the conceptual level. Our studies demonstrated that a single intracardiac injection of a retroviral vector–containing AT$_1$R-antisense (AT$_1$R-AS) or angiotensin-converting enzyme (ACE)–antisense cDNA prevents the development of hypertension in the spontaneously hypertensive (SH) rat on a long-term basis.4–8 This sustained antihypertensive effect of antisense gene therapy was not accompanied by visual side effects and was associated with long-term expression of the AT$_1$R-AS transcript and decreased numbers of AT$_1$R in Ang II–targeted tissues.5 During the investigation, we noticed that despite a robust and comparable expression of AT$_1$R-AS, this strategy had no effect on the basal blood pressure (BP) of normotensive rats,4–6 leading us to hypothesize that the involvement of the RAS in the control of normal BP is limited. However, the contribution of the RAS is of greater significance during the development, establishment, and maintenance of hypertension. If our hypothesis is correct, we argued, then one should expect that the AT$_1$R–expressing normotensive rat would be protected from hypertension. The objective of the present study was to evaluate our hypothesis with chronic low-dose Ang II infusion in a rat model of hypertension.

Materials and Methods

Treatment of Rats With LNSV-AT$_1$R-AS

Pregnant female Sprague-Dawley (SD) rats were obtained from the breeding colony at Wake Forest University, Winston-Salem, NC. For the AT$_1$R-AS study, female offspring were removed from their mothers 5 days after birth and divided into 3 groups: saline control, retroviral (LNSV) vector control, and LNSV vector containing AT$_1$R-AS cDNA (LNSV-AT$_1$R-AS), as previously described.3,6 AT$_1$R-AS (nucleotides –132 to +1128) was chosen because previous studies demonstrated that it blocked the functional aspects of both AT$_1$A and AT$_1$B receptor subtypes.9 Intracardial delivery of LNSV-AT$_1$R-AS, saline, or LNSV (viral control) was carried out in 5-day-old rats.4–6 At age 10 weeks, animals were ovariecetomized and fitted with ALZET 2004 minipumps (ALZET Corporation). Animals were lightly anesthetized with methoxyflurane (Metofane, Mallinckrodt Veterinary, Inc), small incisions were made in the back, minipumps filled with physiological saline or Ang II were fitted, and ALZET pumps were inserted into a subcutaneous pocket in the neck region. At age 14 weeks, rats were ovariectomized and fitted with ALZET 10024 minipumps (ALZET Corporation).”
inserted just below the skin between the shoulder blades, and the incisions were closed with wound clips. Animals were then returned to their individual cages. The pumps delivered 0.25 \mu L/h for 4 weeks for a final dose of Ang II of 55 ng \cdot kg^{-1} \cdot min^{-1}.

### Physiological Measurements

Indirect BPs were monitored weekly for 4 weeks with the use of the tail-cuff method. At the end of this time period, the effect of Ang II on dipsogenic response was determined, as previously described. Water intake was expressed as milliliters per kilogram of body weight.

Direct BP measurements were carried out in cannulated rats. The animals were anesthetized with a rodent cocktail containing ketamine (100 mg/mL) and xylazine (20 mg/mL), which was administered intramuscularly (0.7 mg/kg). Jugular vein and carotid artery were catheterized for direct BP measurements in free-moving, nonrestrained animals, with a pressure transducer coupled to a Digi-Med BP analyzer (Micro-Med). After a 30-minute equilibration period, the pressor response to Ang II (0.02 to 0.32 58 g/kg IV) was determined. The effect of losartan (10 mg/kg) on BP also was examined similarly. After the Ang II pressor response, cannula flushing with 0.9% physiologic saline was administered intravenously as a control. Animals were then administered losartan intravenously, and the systolic BP was determined. Contractile responses of thoracic aorta and renal arterioles to Ang II were measured, as previously described.

### Biochemical Measurements

A semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was used to measure the levels of AT1R-AS transcript and the Neo\(^{5}\) gene in various Ang II target tissues. Total RNA was subjected to a reverse-transcription reaction with the use of an AT1R sense primer (5'-CTCTCTCTCAATCTCCTGG-3') This procedure was followed by 33 cycles of PCR using both sense and antisense primer (5'-CCAGAAACGGCGTAGAAGCCAGGG-3'). PCR products were analyzed by polyacrylamide gel electrophoresis. The sense primer used to detect the Neo\(^{5}\) gene expression was 5'-CGATCTGATCAAGACAGGAT-3' and the antisense primer was 5'-GCCACAGTCGATGAATCCAGAA-3'.

### RNAse Protection Assay

RNAse protection assay was used to detect viral AT1R-AS mRNA by synthesizing a sense probe using AT1R cDNA following manufacturer's protocol (Ambion). Genomic DNA was isolated and purified, and a PCR step was performed for generating a template for the synthesis of the labeled probe by adding a T7 promoter sequence to a 5' upstream primer in the following manner: 5'-GGATCCTAATACGACTCACTATAGGGAGATGGCCCTTAACTCTTT; 3'-CTGCGTAGAGGTTAGCAGCTCA. This step enabled the direct incorporation of the T7-promoter sequence into the template. This template was used in the in vitro reaction to synthesize the mRNA with \(^{32}\)P-CTP in the transcription reaction containing unlabeled 10 mmol/L ATP, 10 mmol/L UTP, 10 mmol/L GTP, and 100 \mu mol/L CTP and RNA polymerase. The reaction was incubated at 37°C for 1 hour and then treated with DNase I. The labeled probe was then gel purified and used within 24 hours. In the hybridization reaction, the labeled probe (4 \times 10^6 cpn) was hybridized with 30 \mu g of various RNAs. The RNA was precipitated with ammonium acetate followed by ethanol. Pelleted RNA was dissolved in hybridization buffer and incubated overnight at 42°C. The next day, the reaction was treated with 1:100 dilution of RNaseA/RNaseT1 mix in the digestion buffer. After centrifugation, the pellet was dissolved in the gel-loading buffer and ran on a 5% acrylamide gel. The gel was then exposed to film overnight at –80°C.

### Statistics

All results are expressed as mean±SE. Indirect BP measurements were analyzed by repeated-measures ANOVA. Direct mean BPs were analyzed by ANOVA. Values of P≤0.05 were considered statistically significant. All experiments had n=7 per group, unless otherwise indicated.

### Results

**Characterization of AT1R-AS–Treated SD Rats**

Administration of LNSV-AT1R-AS viral particles to 5-day-old SD rats resulted in the expression of AT1R-AS transcript as early as 10 days after injection. This expression was maintained in various Ang II target tissues throughout the duration of the experiment because it was apparent at 100 days after injection. Representative RT-PCR data from a 14-week-old SD rat are presented in Figure 1. Expression of the AT1R-AS and the Neo\(^{5}\) gene transcript was found in the various peripheral tissues, as shown in Figures 1A and 1B, respectively. Expression of the AT1R-AS and Neo\(^{5}\) gene
transcripts were also observed in several brain areas, including the cerebrum, hypothalamus, and brainstem (Figures 1C and 1D). These observations show that a single intracardiac administration of LNSV-AT1 R-AS in a 5-day-old rat causes high levels of AT 1 R-AS transduction in both the periphery and the brain throughout life. RNAse protection assay was used to confirm RT-PCR data. Figure 2 demonstrates a ~350-bp band corresponding to a protected AT 1 R-AS transcript. 3T3 cells infected with the LNSV-AT 1 R-AS (positive control) demonstrated a robust expression of the transcript, whereas control cells did not show this band.

Despite the long-term expression of the AT 1 R-AS, no significant difference was observed between basal BP in LNSV-AT, R-AS–treated rats (105 ± 2.0 mm Hg) and control rats (saline or LNSV, 109 ± 1.0 mm Hg; Figure 3A). Previous studies have implicated a protective role of estrogens on the BP.10 –12 Additionally, the estrous cycle influences the responsiveness to Ang II.13–15 Therefore, we ovariectomized female rats to eliminate such a protective effect and the cyclic response to Ang II. Figure 3B shows that ovariectomy in adulthood has no effect on basal BP in either LNSV-treated or LNSV-AT 1 R-AS–treated rats. Thus, all subsequent AT 1 R-AS experiments were performed in ovariectomized rats.

Next, we studied the physiological consequence of AT 1 R-AS expression. Ang II is a potent dipsogenic hormone, and a single subcutaneous dose of Ang II (150 μg/kg) in LNSV-treated control rats caused an average water intake of 11 ± 1.5 mL · kg⁻¹ · h⁻¹ (n = 7) compared with an intake of 4.5 ± 1.5 mL · kg⁻¹ · h⁻¹ after administration of saline. The drinking response to the same concentration of Ang II was 4.4 ± 1.5 mL · kg⁻¹ · h⁻¹ (n = 7) in the LNSV-AT, R-AS–treated rats, indicating a complete attenuation of the dipsogenic response by the antisense treatment (Figure 4). Amount of water intake was not different from the amount observed in the same animals after saline administration (1 μL/kg).

Figure 5 represents the effect of acute Ang II administration on changes in systolic BP. Increasing doses of Ang II caused a significantly greater change in systolic BP in LNSV-treated rats compared with LNSV-AT, R-AS–treated rats, demonstrating the reduced response to acute peripheral Ang II. This difference was more pronounced at lower doses of Ang II than at higher doses. The in vitro vascular response demonstrated a similar attenuation to Ang II. Ang II caused a dose-dependent increase in contraction of both thoracic aorta and renal arterioles with a similar EC₅₀ of 43 nmol/L in the LNSV-treated and LNSV-AT, R-AS–treated rats (Figure 6). However, the maximal contraction was decreased by 40% in
LSNV-AT1,R-AS–treated rats (Figure 6). Specificity of the Ang II effect is shown by the fact that KCl- or phenylephrine-induced contraction and acetylcholine-mediated relaxation were not different between the tissues from the LNSV-treated and LNSV-AT1,R-AS–treated rats (data not shown). Collectively, these observations demonstrate that AT1 R-AS expression in SD rats reduced Ang II–induced physiological responses without any influence on basal BP. This reduced physiological responsiveness may be associated with a decrease in the AT1 R number, which has previously been shown in AT1 R-AS–treated rats. 6

Effect of Chronic Ang II Infusion in LNSV-AT1,R-AS–Treated Rats

Because acute Ang II–induced BP and dipsogenic responses were significantly attenuated with antisense treatment, we investigated whether Ang II–induced hypertension would also be affected in the LNSV-AT1,R-AS–treated rat. Figure 7 shows that 55 ng · kg⁻¹ · min⁻¹ infusion of Ang II caused a gradual increase in BP, which reached a maximum of 128±5 mm Hg in 4 weeks in LNSV-treated rats (Figure 7A).

However, the Ang II infusion failed to increase BP in LNSV-AT1,R-AS–treated rats, with BP similar to that observed in the ovariectomized control without the Ang II pump. A similar difference in the effect of Ang II was observed when direct BP was measured; ie, BP in LNSV-AT1,R-AS–treated rats was significantly lower than in LNSV-treated rats and controls (Figure 7B). Intravenous administration of losartan (10 mg/kg) in chronically Ang II–treated rats caused a 13±4 mm Hg decrease in BP in LNSV-treated rats, whereas the same dose of losartan resulted in a 6±2 mm Hg decrease in the BP of LNSV-AT1,R-AS–treated rats (Figure 7C).

Cardiac hypertrophy is a hallmark of hypertension. Ang II–infused LNSV-treated rats showed a 65% increase in the ratio of heart weight to body weight (HW/BW; Figure 8). This hypertrophy was significantly reduced in the LNSV-AT1,R-AS–treated rats. Ang II–induced contraction of the

Figure 5. Changes in systolic BP (SBP) in response to acute Ang II in LNSV-AT1,R-AS–treated SD rats. Rats treated with LNSV or LNSV-AT1,R-AS at 5 days of age were implanted with catheters at 14 weeks of age, as described in Materials and Methods. Mean change in systolic BP from basal levels to indicated concentrations of Ang II was determined in unanesthetized rats. Data are mean±SEM; n=7. *P<0.05 vs LNSV.

Figure 6. Effect of LNSV-AT1,R-AS on contractility of renal artery and abdominal aorta. Three-millimeter ring segments were cut and cleaned of adhering fat and tissue. Rings were mounted, and their contractility was measured in response to increasing concentrations of Ang II. For renal artery, rings were washed multiple times with Kreb's buffer between doses to avoid tachyphylaxis. n=6.

Figure 7. Effect of chronic Ang II infusion on indirect SBP in ovariectomized SD rats treated with LNSV-AT1,R-AS. Osmotic minipumps were implanted, as described in Materials and Methods, and delivered 55 ng · kg⁻¹ · min⁻¹ over 4 weeks. Indirect BPs were measured weekly by tail-cuff method. Data are mean±SEM; n=7. *P<0.001 vs LNSV. B, Effect of LNSV-AT1,R-AS treatment on direct SBP in ovariectomized SD rats treated with chronic Ang II. Five-day-old SD rats were injected with LNSV or LNSV-AT1,R-AS and were allowed to grow for 70 days, and then osmic minipumps were implanted for 4 weeks. After this time period, pumps were removed and animals were implanted with catheters in carotid artery to measure direct BP. *P<0.05 vs control; **P< vs LNSV. C, Effect of losartan on direct SBP in LNSV-AT1,R-AS–treated SD rat. Losartan was injected at a dose of 10 mg/kg IV and SBP measured 1 hour later. Data are mean±SEM; LNSV, n=6; antisense, n=7. *P<0.05.
renal artery was increased in LNSV-treated rats after chronic Ang II infusion. This increase was attenuated in the LNSV-AT1R-AS–treated rats such that it was comparable to renal artery of control rats that were not infused with Ang II (data not shown). These observations demonstrate that, in addition to protection from high BP, there was a significant improvement in cardiac and renovascular responsiveness in the AT1R-AS–treated rats.

Discussion

The most significant finding of the present study is that pretreatment with antisense targeting the RAS protects normotensive rats from developing Ang II–dependent hypertension. As a result, the antisense gene therapy strategy proves useful in the treatment and prevention of hypertension. Our studies previously demonstrated that interruption in the expression of ACE or the AT1R prevents the SH rat from developing hypertension and associated cardiac and renovascular remodeling, 2 hallmarks of hypertension. In the present study, single administration of a bolus of LNSV-AT1R-AS in a 5-day-old rat resulted in a significant level of AT1R-AS expression, as measured by both RT-PCR and RNAse protection assay in various Ang II target tissues, such as heart, adrenal, kidney, and liver, an observation consistent with our previous data. The AT1R-AS expression seems to be associated with an inability of Ang II to induce high BP, which is closely possible the development of Ang II–dependent hypertension. Published studies have also demonstrated a similar phenomenon, with infusion of a higher dose of Ang II for a much shorter duration resulting in a similar 45- to 60-mmHg dose-dependent increase in BP. We decided to use a low subpressor dose for a longer time period to allow observation of any effects of the antisense in this model and to mimic as closely as possible the development of Ang II–dependent human hypertension. Ang II infusion resulted in a 20- to 30-mmHg increase in BP from the start of infusion in ovarietomized SD females. This increase was prevented completely in ovarietomized SD females treated with AT1R-AS.

Losartan and ACE inhibitors have been shown to cause regression or prevention of cardiac hypertrophy. In the present study, single administration of a bolus of LNSV-AT1R-AS in a 5-day-old rat resulted in a significant level of AT1R-AS expression, as measured by both RT-PCR and RNAse protection assay in various Ang II target tissues, such as heart, adrenal, kidney, and liver, an observation consistent with our previous data. The AT1R-AS expression seems to be associated with an inability of Ang II to induce high BP, which is closely possible the development of Ang II–dependent hypertension. Published studies have also demonstrated a similar phenomenon, with infusion of a higher dose of Ang II for a much shorter duration resulting in a similar 45- to 60-mmHg dose-dependent increase in BP. We decided to use a low subpressor dose for a longer time period to allow observation of any effects of the antisense in this model and to mimic as closely as possible the development of Ang II–dependent human hypertension. Ang II infusion resulted in a 20- to 30-mmHg increase in BP from the start of infusion in ovarietomized SD females. This increase was prevented completely in ovarietomized SD females treated with AT1R-AS.

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Chronic infusion of subpressor doses of Ang II has been shown to cause an increase in AT1Rs, a phenomenon termed autopotentiation effect.25,26 As a result, there is an increased sensitivity to Ang II on BP, which may be attributable to increases in the AT1Rs.25 Our studies demonstrate that AT1-R-AS treatment completely prevents the autopotentiation effect. The mechanism of autopotentiation and its resistance in the AT1-R-AS–treated rats remains unelucidated, but it may be related to a decrease in the levels of AT1Rs in Ang II target tissues.

The mechanisms of hypertension caused by chronic Ang II infusion are not completely understood. The increase in BP can be mediated by a direct action on the vascular smooth muscle to increase total peripheral resistance, actions on the kidney to promote sodium retention, and resistance changes that are mediated by central effects of Ang II. Several investigators have demonstrated that hypertension induced by the use of chronic low-dose Ang II may be mediated through the CNS pathway.27,28 Although our experiments were not designed to identify the precise mechanism of Ang II–dependent hypertension, this study demonstrates for the first time that a systemic administration of AT1-R-AS by a retroviral vector results in its transduction into the brain. Lack of a tight blood-brain barrier in neonatal rats may explain this observation. Expression of the AT1-R-AS transcript was detected in several brain areas, including the hypothalamus, which is known to regulate Ang II–induced dipsogenic response. The dipsogenic response was significantly attenuated in the AT1-R-AS–treated animals, which may be due to a decrease in central AT1R number.

In conclusion, our studies indicate that pretreatment with the LNSV-AT1-R-AS protects normotensive rats from developing Ang II–induced hypertension. In addition, our data demonstrate that viral vector can transport the AT1-R-AS across the poorly defined blood-brain barrier in neonates for its robust and long-term expression in the brain. Thus, these studies provide additional support to our view that antisense gene therapy that targets the RAS is a conceptually sound strategy for the prevention and possible cure of hypertension.

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References


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