Attenuation of Hypertension and Heart Hypertrophy by Adeno-Associated Virus Delivering Angiotensinogen Antisense

Birgitta Kimura, Dagmara Mohuczy, Xiaoping Tang, M. Ian Phillips

Abstract—Angiotensinogen (AGT), one of the major components in the renin-angiotensin system, has been linked to hypertension in humans and animals. We have previously systemically administered antisense oligonucleotides and plasmid vectors with DNA that targeted AGT and attenuated hypertension in spontaneously hypertensive rats. The aim of the present study was to prolong the effect of antisense treatment by the use of a recombinant adeno-associated viral (rAAV) vector targeted to AGT. Using a model of lifelong hypertension in which 5-day-old spontaneously hypertensive rats are treated, a single intracardiac injection of rAAV-AGT-antisense (rAAV-AGT-AS) delayed the onset of hypertension for 91 days and significantly attenuated hypertension in adulthood for up to 6 months. Systolic blood pressure was always lower, by up to 23 mm Hg in the AS-treated group. The vector was stable and expressed a reporter gene in liver, kidney, and heart. The rAAV-AGT-AS treatment significantly decreased left ventricular hypertrophy ($P<0.01$) and also lowered levels of AGT in the liver ($2.78\pm0.61$ µg/g tissue versus $5.23\pm0.41$ µg/g tissue for the sense-treated group, $P<0.01$). Measurement of liver transaminases showed no evidence for liver toxicity. We conclude that rAAV-AGT-AS offers a safe, stable approach for gene therapy of hypertension. (Hypertension. 2001;37[part 2]:376-380.)

Key Words: adeno-associated virus ■ hypertension, cardiac ■ antisense ■ gene therapy ■ angiotensinogen

Antisense inhibition for gene therapy of hypertension is a novel approach to provide long-term control of hypertension.1 Because the antisense strategy can target genes precisely, inhibition of specific proteins can be achieved. Although the genes involved in hypertension are largely unknown, currently used drugs control high blood pressure by inhibiting a few proteins such as angiotensin-converting enzyme (ACE), $\beta_1$-adrenoceptors ($\beta_1$-AR), and angiotensin type 1 receptor (AT$\,\,^1$R). Therefore, specific gene targeting of these proteins has been our strategy with antisense oligodeoxynucleotides (AS-ODNs). Compared with current drugs, AS-ODNs are more specific and longer lasting and therefore may have certain advantages over current drug therapy. The longer duration of action could provide more consistently effective blood pressure control and thereby increase patient compliance. Despite all the drugs available, only $\approx29\%$ of all hypertensives have their high blood pressures controlled. Therefore, there is a need to explore a new generation of methods of treatment. Gene therapy, while oversold, still offers an approach that could produce prolonged benefits if based on scientific research. Whereas AS-ODN can reduce high blood pressure in rat models for weeks with a single dose,1,2 even longer-lasting effects can be achieved with viral vectors.3 Studies with the retrovirus used to deliver antisense to AT$\,\,^1$R or ACE have been shown to produce long-lasting effects in reducing high blood pressure when given in the first 5 days after birth.3,4 Retroviruses, however, have disadvantages as a vector for therapeutic use. These include the possibility of tumorigenic activity, the vector entering the germ line, and the limitation that retroviruses only infect dividing cells. Adeno-associated virus (AAV) has the potential to provide stable, effective, and very long-lasting delivery of antisense,5 even in nondividing cells. Because the previous studies have targeted the AT$\,\,^1$R for inhibition of the renin-angiotensin system (RAS), the purpose of this study was to target angiotensinogen (AGT) mRNA with antisense and deliver it with AAV as a vector. AGT has been shown to be elevated in some patients with hypertension and is a critical component of the RAS. We hypothesized that antisense targeted to AGT mRNA, delivered by AAV, will produce a prolonged, stable effect and reduce high blood pressure in spontaneously hypertensive rats (SHR). Thus, the purpose of this study was to determine for the first time the duration and magnitude of antihypertensive effects of AAV-AS targeting AGT. This study establishes the AAV as a potential vector for the delivery of antisense. The study also shows that a single systemic injection of AAV-AS-AGT consistently attenuates high blood pressure for many months.
Methods

Animals
Five-day-old male SHR were obtained either as pups of rats from Charles River SHR bred at the University of Florida or as 2-day-old pups accompanied by their dam from Charles River. They were kept with their dam until 21 days of age. At 5 days of age, the pups were anesthetized with Metofane and intracardially injected with $1 \times 10^6$ infectious particles of recombinant AAV-AGT-AS (rAAV-AGT-AS, $n=15$) or rAAV-AGT-S ($n=10$) constructs in a volume of 25 μL. Rats were kept in a temperature-controlled room on a 12-hour day/night cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Adeno-Associated Viral Constructs
The AAV constructs contain full-length rat tissue AGT in either the antisense or the sense orientation. The plasmid construct is shown in Figure 1. The reporter gene was green fluorescent protein (GFP). The cDNA containing the AGT sequence was a gift from Dr Lynch, University of Virginia, Charlottesville, Va. Details on the construction and production of the plasmids have been reported previously.6 The plasmids were packaged into AAV-2 by the Gene Therapy Center at the University of Florida.

Experimental Protocol
Systolic blood pressure was measured every week, starting at 7 weeks of age, using the tail-cuff method (Narco Biosystems). Blood samples for AGT measurements were withdrawn from the tongue vein under Metofane anesthesia at 7 weeks of age and every 3 to 6 weeks thereafter. Blood samples were obtained 24 to 48 hours before blood pressure measurement. After 26 weeks, animals were deeply anesthetized with 30 mg ketamine/6 mg xylazine/1 mg acepromazine per kg bodyweight given subcutaneously and perfused with ice-cold saline via the left ventricle. The left ventricle was dissected out and weighed. The left ventricular weight in grams divided by the body weight in kilograms was used as a measure of left ventricular hypertrophy. A piece of liver, one quarter of the kidneys, and a piece of left ventricle, brain stem, and hypothalamus were dissected out and frozen on dry ice for AGT measurement. Tissues from 3 animals from the sense and 5 from the antisense construct–treated group were obtained at different times after injection. Pieces of tissue were taken from a 14-week-old rat for DNA measurements.

Detection of the Transgene in Rat Tissues
Fourteen weeks after intracardiac injection, tissues were harvested and DNA isolated using TRI Reagent. Presence of the transgene GFP was analyzed by nested polymerase chain reaction (PCR). One microgram of the DNA was used for each 50 μL reaction. The first set of primers consisted of the forward primer 5′-CAGCGGAGAGGGTGAAGGTG and the reverse primer 5′-CAGGGCAGACTGGGTGGACA-3′. The PCR conditions were 94°C for 5 minutes, then 94°C for 45 seconds, 60°C for 45 seconds, and 68°C for 1 minute for 35 cycles. Two microliters from the first PCR reaction were used for the second reaction. The second set of primers consisted of forward primer 5′-GCCACATACGG-L and reverse primer 5′-ATGGTTGTCTGGGAGGAC-3′ under the same PCR conditions. The expected product size was 489 bp. The template for the positive PCR control was a GFP-containing plasmid. Amplification products (10 μL) were analyzed on 1% agarose gel stained with ethidium bromide.

Angiotensinogen and Liver Transaminase Measurements
AGT was extracted from tissues in Tris buffer, the pH was adjusted to 4.5, and from the supernatant obtained after centrifugation used for the radioimmunoassay of AGT.7 Plasma was diluted to the appropriate concentrations immediately before the assay. Pure rat AGT and the antibody against AGT were gifts from Dr Conrad Sernia, University of Queensland, Brisbane, Australia. Alanine aminotransferase and aspartate aminotransferase were measured by a kit from SIGMA Diagnostics.

Statistical Analysis
Values are expressed as mean±SEM. Differences in blood pressure and plasma AGT between the 2 treatment groups were analyzed by 2-way ANOVA repeated in 1 dimension followed by the Student-Newman-Keuls multiple range test or 1-way ANOVA. Differences between protein levels in tissue and in left ventricular hypertrophy were analyzed by Student’s t test. P<0.05 was considered significant.

Results

Blood Pressure
The effect of a single intracardiac injection of rAAV-AGT constructs on blood pressure is shown in Figure 2. There was no difference in the systolic blood pressure of the AGT antisense–construct injected group versus the sense-construct injected group until 10 weeks of age. Up to that point, both treatment groups were normotensive. As hypertension developed (ie, >150 mm Hg), the antisense-treated group had significantly lower systolic blood pressures than the sense-treated group (P<0.001). This difference lasted from 10 weeks of age to the end of the experiment at 26 weeks of age. The maximum difference in blood pressure did not exceed 25 mm Hg but persisted throughout the 6-month study period. Although the antisense-treated group developed hypertension, the onset was delayed and it was less severe than the hypertension of the sense-treated group.

Hypertrophy
Figure 3 shows the difference in left ventricular hypertrophy between the different treatments. Treatment with the AGT antisense construct significantly attenuated left ventricular hypertrophy in SHR at 26 weeks of age. The left ventricular weight/body weight ratio was 2.99±0.04 (n=7) in the antisense-treated group versus 3.16±0.04 (n=6) in the sense-treated group (P=0.01). The degree of hypertrophy in the
sense-treated group was similar to that of control 26-week-old SHR (3.20 ± 0.02, n = 3).

Toxicity
Liver transaminases were used as a measure of possible toxicity of the rAAV. Both ALT and AST levels were normal and did not differ between the 2 treatment groups. Normal plasma levels in SHR range from 60 to 80 U/L for AST and 15 to 25 U/L for ALT (unpublished data). The results are summarized in Table 1. All of the rat pups injected with the viral construct in this study survived and appeared healthy throughout the experiment.

Detection of Viral Vector
The detection of GFP DNA 14 weeks after injection was used as a measure of the presence of the rAAV construct. The GFP gene is situated downstream from the AGT gene and can thus be used as a marker for the presence of the AGT gene (Figure 1). We could detect GFP DNA by PCR in the liver, kidney, heart, and brain (Figure 4). The strongest signal was observed in the liver, the organ in which the majority of AGT is produced.

Angiotensinogen Levels
We measured AGT levels in plasma and different tissues to determine the efficacy of the rAAV-AGT antisense construct. Treatment with the AGT antisense construct significantly lowered the levels of AGT in the liver at 26 weeks of age (P = 0.007) as shown in Figure 5. The AGT concentration was 2.78 ± 0.61 µg/g tissue in the antisense construct–injected group versus 5.23 ± 0.41 µg/g tissue in the sense construct–injected group. The levels in the sense-treated group were comparable to those of adult SHR (5.34 ± 0.73 µg/g tissue). The levels in the antisense-treated group were lower than both adult SHR and adult Wistar-Kyoto rats (4.04 ± 0.46 µg/g tissue). There was a slight decrease in the AGT levels in the kidney but the difference between the groups was not significant. There were no significant differences between the 2 treatment groups in the left ventricle, brain stem, or hypothalamus. The results from tissues are summarized in Table 2. Plasma levels, shown in Figure 6, did not change significantly over time in the sense-treated group, but there was a significant decrease with time in the antisense group. At 26 weeks of age, AGT levels in the sense-treated group were higher than in the antisense-treated group.

Discussion
Our results show that a single injection of rAAV-AGT-AS given systemically at 5 days of age can attenuate the development of hypertension in SHR and reduce the level of high blood pressure in adulthood by 20 mm Hg. The effect was highly consistent and very prolonged. The antisense-treated group never attained as high a systolic blood pressure as the sense group, even up to 6 months after the injection. It
is possible that higher doses of the viral construct would have had larger effects on blood pressure.\(^3\) A current limitation is the small scale production of a high-titer AAV vector. It is also possible that other mechanisms involved in the regulation of blood pressure in the SHR may resist full normalization of blood pressure. One such mechanism could be via the central RAS, because it has been shown to be upregulated in the SHR.\(^8\) The sympathetic nervous system is also involved, and disruption of this system in the early postnatal period attenuates hypertension in SHR.\(^9\)

In addition to attenuating hypertension, the rAAV-AGT-AS also decreased the degree of left ventricular hypertrophy. The RAS has been hypothesized to provide angiotensin II as a growth factor that increases cardiac hypertrophy.\(^10,11\) A decrease in AGT levels should thus decrease the degree of hypertrophy, which is, indeed, what we found. But we also reduced the blood pressure and therefore the afterload on the heart, which could result in decreased hypertrophy.

![Figure 4. The transgene expression in rat tissues. Five-day-old SHR were injected into the heart with rAAV-AGT-AS-IRES-GFP. DNA was isolated from the tissues 14 weeks later, and expression of the GFP was determined by nested PCR, as described in Methods.](image)

![Figure 5. AGT levels in the liver of 26-week-old rats, half a year after intracardiac injection of rAAV-AGT-AS or rAAV-AGT-S. n=9 for antisense, n=7 for sense. The difference between the groups is significant, P<0.01.](image)

![Figure 6. Plasma AGT levels after a single intracardiac injection of rAAV-AGT-AS or rAAV-AGT-S in 5-day-old SHR. Two-way ANOVA revealed a significant difference between the treatments with time, P<0.01. One-way ANOVA showed no significant difference with time in the sense construct–treated group, P=0.219, but a significant decrease over time in the antisense construct–treated group, P<0.001. Solid bar indicates rAAV-AGT-S, n=7 to 10; shaded bar, rAAV-AGT-AS, n=8 to 15.](image)

We were able to detect GFP DNA, which was used as a proxy measure for our construct in the liver, kidney, heart, and brain. AAV has not previously been reported to access the brain from the periphery. Thus, the presence in the brain was unexpected but may be due to an open blood-brain barrier at the time the animals were injected. In rats, the blood-brain barrier matures at 1 to 2 weeks of age,\(^12\) and as we injected 5-day-old rat pups, there could have been leakage of viral vector into the brain.

The major test of whether antisense treatment inhibits the system it is aimed toward is to determine whether it changes the concentration of the targeted protein. We measured both plasma AGT levels at intervals during the experiment and tissue AGT levels at the end of the study, half a year after injection. We found a significant decrease in the AGT levels in the liver, indicating that the rAAV-AGT-AS was effective in the tissue in which the majority of AGT is synthesized. There was no difference in the levels between the 2 treatment groups in the brain, which points to it not affecting the central RAS, probably because of limited access to AGT-synthesizing cells. The AGT-AS group showed a significant decrease of AGT with time in the plasma but not the sense-treated group. The decrease was not evident at 10 and

<table>
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<th>Tissue</th>
<th>AGT-Sense</th>
<th>AGT-Antisense</th>
<th>P Value</th>
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<tr>
<td>Brainstem</td>
<td>1.28±0.40</td>
<td>1.00±0.36</td>
<td>0.613</td>
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<td>Hypothalamus</td>
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<td>1.29±0.31</td>
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<td>Liver</td>
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<td>2.78±0.61</td>
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<tr>
<td>Kidney</td>
<td>1.68±0.36</td>
<td>1.18±0.55</td>
<td>0.446</td>
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<tr>
<td>Left ventricle</td>
<td>1.53±0.22</td>
<td>1.41±0.12</td>
<td>0.603</td>
</tr>
</tbody>
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Data are mean±SEM. The unit for AGT is \(\mu g/g\) tissue. *indicates P<0.01.
15 weeks after the injection, although at this time the blood pressure showed significant differences between groups. We hypothesize that the early effects on blood pressure are due to decreases in tissue AGT (eg, kidney). There may also be a transient increase in AGT production over time to counteract the effect of the rAAV-AGT-AS. Pedrazzini et al., for example, found that in transgenic mice producing AGT antisense, there was an increase in AGT levels both in liver and plasma at 10 to 14 weeks after an initial decrease.

One of the concerns with use of viral vectors is their potential toxicity. To address this, we measured liver transaminases in the plasma at the end of the experiment. Our results showed no evidence for liver toxicity, which implies that the viral vector we used is nontoxic. Although we cannot rule out a transient immune response or short-term cellular toxicity, our results show that there are no long-term toxic effects on the liver because the measurements of transaminases were performed 25 weeks after injection.

The results are similar to the effect of injecting a retro-viral vector to deliver AS to AT1 receptors in 5-day-old SHR reported by Martens et al., who found that hypertension was prevented from developing for 92 days. Our results show a significant effect in slowing the increase in systolic blood pressure for 84 days, but we did not prevent hypertensive levels from being reached. This may reflect a difference in vector dose or the weaker effect of AGT versus AT1-R as the target for antisense. However, there are important differences between the viral delivery approaches. Retroviruses can only infect dividing cells, therefore they have to be used in neonatal rats. AAV infects both dividing and nondividing cells and can be used in young or adult animals. Therefore, the AAV delivery of AS will be the most appropriate vector for reversing hypertension in adults. The present study serves to demonstrate that AAV can be used as an effective vector for antisense delivery in models of hypertension.

In summary, rAAV-AGT-AS delayed the development of hypertension in young SHR and persistently reduced blood pressure in adulthood for up to 6 months after a single, systemic injection. There was also reduced left ventricular hypertrophy but no evidence of liver toxicity half a year after administration of the viral vector. We conclude that rAAV-AGT-AS offers a safe, stable approach for gene therapy of hypertension.

Acknowledgment

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References

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