Intravenous angiotensinogen antisense in AAV-based vector decreases hypertension

XIAOPING TANG, DAGMARA MOHUCZY, Y. CLARE ZHANG, BIRGITTA KIMURA, SARA M. GALLI, AND M. IAN PHILLIPS

Department of Physiology, College of Medicine, University of Florida, Gainesville, Florida 32610

Intravenous angiotensinogen antisense in AAV-based vector decreases hypertension. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2392–H2399, 1999.—Angiotensinogen (AGT) has been linked to hypertension. Because there are no direct inhibitors of AGT, we have developed antisense (AS) inhibition of AGT mRNA delivered in an adeno-associated virus (AAV)-based plasmid vector. This plasmid, driven by the cytomegalovirus promoter, contains a green fluorescent protein reporter gene and AS cDNA for rat AGT. Transfection of the plasmid into rat hepatoma cells brought a strong expression of the transgenes and a significant reduction in the level of AGT. In the in vivo study, naked plasmid DNA was intravenously injected into adult spontaneously hypertensive rats at different doses (0.6, 1.5, and 3 mg/kg). Expression of AGT AS mRNA was present in liver and heart, and it lasted longer in the liver. All three doses produced a significant decrease in blood pressure (BP). BP decreased for 2, 4, and 6 days, respectively. The lowest dose decreased BP by 12 ± 3.0 mmHg, whereas the higher doses decreased BP by up to 22.5 ± 5.2 mmHg compared with the control rats injected with saline (P < 0.01). The injection of the plasmid with liposomes produced a more profound and longer reduction (8 days) in BP. Consistent changes in plasma AGT level were observed. Sense plasmid had no effect. No liver toxicity was observed after injection of AS plasmid with or without liposomes. Our results suggest that the systemic delivery of AS against AGT mRNA by AAV-based plasmid vector, especially with liposomes, may have potential for gene therapy of hypertension and that further studies with the plasmid packaged into a recombinant AAV vector for a longer-lasting AS effect are warranted.

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MATERIALS AND METHODS

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure (BP). Some specific hormones in this system act to constrict blood vessels and increase the renal retention of sodium and water, therefore resulting in an increase in BP (33). Angiotensinogen (AGT), produced largely in the liver, is cleaved by renin to ANG I, which is further cleaved by ANG-converting enzyme (ACE) to ANG II, a potent vasoconstrictor and regulator of BP (18, 31). Human hypertension has been linked to a mutation in the AGT gene that leads to overexpression of AGT. AGT has been shown to be an important determinant of human BP (15). Overactivity of the AGT gene has also been implicated in the development and maintenance of hypertension in rat models of essential hypertension, including spontaneously hypertensive rats (SHR; see Refs. 11 and 42), and in transgenic mice (10, 16, 25). There are several ways to inhibit the components of the RAS, including ACE inhibitors, renin inhibitors, and ANG II antagonists. However, there is no specific drug in use as a direct inhibitor for AGT.

Antisense (AS) molecules have been used to successfully inhibit protein synthesis in a number of biological systems (5, 24, 29). They can bind to a complementary region of target mRNA and block selected gene expression without changing the functions of other genes (13). Therefore, AS strategy may be a good choice for a gene therapy approach to inhibit AGT. AS DNA can be designed in a plasmid vector with a promoter to drive a stronger expression. In the present study, we report the design and use of cytomegalovirus (CMV) promoter-driven AS against AGT mRNA delivered in an adeno-associated virus (AAV)-based plasmid vector, pAAV-AGT-AS, tested both in vitro and in vivo. Studies using recombinant plasmids containing AAV inverted terminal repeats (ITRs) have attracted considerable interest lately. AAV-based plasmids have been shown to drive higher and longer transgene expression than the identical plasmids lacking the ITRs of AAV in most cell types (7, 28, 37, 39). In this paper, we report that delivery of AS to AGT by pAAV-AGT-AS vector resulted in a significant decrease in the levels of secreted AGT in vitro and decreases in plasma AGT levels and reduction of the high BP of SHR in vivo.

Construction and production of plasmid. A 1.65-kb full-length cDNA of rat tissue AGT was released from the pRang 6 plasmid DNA (19) by Kpn I/Xba I restriction enzyme digestion. The released DNA fragment was subcloned into the Hind III site of the AAV-derived eukaryotic expression vector pTR-UF3 (provided by N. Muzyczka, University of Florida) by blunt-end ligation in the AS orientation (pAAV-AGT-AS). As a control, a plasmid with the AGT gene inserted in the sense direction (pAAV-AGT-S) was constructed. The newly resulted AAV-based bicistronic plasmid is shown in Fig. 1. The cassette between two AAV terminal repeats (AAV ITRs) contains CMV promoter, AGT, the polio virus type 1 internal ribosomal entry site (IRES), and green fluorescent protein (GFP) gene. AAV ITRs may have promoter activity. They may facilitate the plasmid’s entry into host cells and increase the transgene expression. They are essential for the plasmid to be packaged into recombinant AAV (rAAV) and for AAV integration. IRES allows separate expression of the AGT and GFP gene. The orientation and sequence of the insert were confirmed by both restriction enzyme digestion and direct sequencing.

Plasmid DNA was prepared from cultures of the plasmid-transformed Escherichia coli (SURE 2) by using Qiagen kits.
Fig. 1. Diagram of rat angiotensinogen (AGT) adeno-associated virus (AAV)-based bicistronic plasmid constructs. Plasmid (p) AAV-AGT-AS and pAAV-AGT-S were constructed by inserting a full-length cdNA (1.65 kb) of AGT into the unique Hind III site of the AAV-derived plasmid pTR-UF3 in antisense (AS) or sense (S) direction, respectively. AGT cdNA, indicated by shaded arrow, is driven by a cytomegalovirus (CMV) early promoter (Pcmv). ITR, AAV inverted terminal repeat; IRES, polio virus type 1 internal ribosomal entry site; h-GFP, “humanized” Victoria green fluorescent protein gene. Other open bars and arrows represent other basic elements of the vector.

according to the manufacturer’s directions. The purified DNA was reconstituted in sterile saline (0.9% sodium chloride) with the concentration of 2 mg/ml.

Cell culture. Reuber hepatoma cells, H4-II-E, were purchased from ATCC (Rockville, MD). Cells were grown in DMEM containing 2 mM L-glutamine and 4.5 g/l glucose, supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were kept in an incubator (Quene policeman. The cell lysates were then diluted with distilled water and used for protein measurement by Bradford (4) assay using protein reagent from Bio-Rad (Hercules, CA).

Animals. Adult male SHR (18 wk old, body wt 300–350 g) were used (Harlan, Indianapolis, IN). They were housed at a constant room temperature with a 12:12-h light-dark cycle and were fed with standard laboratory rat chow and tap water ad libitum. All experimental procedures were approved by the Institutional Animal Uniform Care Committee of the University of Florida, Gainesville.

Intravenous DNA delivery. Rats were anesthetized by metofane inhalation. A 27-gauge needle linked to a 3-ml syringe was used for the tongue vein injection. The first experiment was to test the effectiveness of naked plasmid DNA and the dose-response relationship. SHR were injected with plasmid DNA at the dose of 0.6, 1.5, and 3 mg/kg. The rats injected with saline or naked sense plasmid served as the controls. To compare the effect of injection of AS with and without liposomes, the following experiment was performed.

In this experiment, SHR were injected with 3 mg/kg of pAAV-AGT-AS DNA alone or pAAV-AGT-AS mixed with liposomes composed of 1,2-dioleoyl-3-trimethylammonium propane and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine at a molar ratio of 1:1 (provided by J. Hughes, College of Pharmacy, University of Florida; see Ref. 34). A ratio of 1:3 (wt/wt) of DNA-liposomes was used. The rats injected with liposomes only or pAAV-AGT-S complexed with liposomes served as the controls. The volume of injection in the first experiment was 1 ml. In the second experiment, a volume of 4 ml was used not to make any visible precipitation in the mixture.

Blood sample collection. For each rat, 500 µl of tail blood were taken before injection and on days 3, 5, and 7 after injection. Blood was collected into prechilled tubes containing 30 µl of 0.3 M EDTA. Plasma was separated and stored at −80°C until measurements were made.

BP measurement. Systolic BP (SBP) of SHR was measured with a Programmed Electro-Sphygmomanometer PE-300 (Narco Bio-Systems, Division of International Biomedical, Austin, TX) using the tail-cuff method. Nonanesthetized rats were placed in a plastic restraining holder mounted on a thermostatically controlled warm plate that was maintained at 37°C during measurement. An average of 10 readings was taken for each animal.

Detection of transgene mRNA in tissues of SHR by RT-PCR. To detect the expression of AS AGT mRNA in SHR after plasmid delivery, another two SHRs were injected with pAAV-AGT-AS DNA alone or liposomes along with pAAV-AGT-AS DNA into the tongue veins.
3 mg/kg of naked pAAV-AGT-AS and killed by decapitation on days 3 and 7, respectively. Total RNA was isolated from heart, liver, and kidney using Trizol reagent (GIBCO-BRL). RNA (5 µg) was used for RT-PCR, as described above for H4 cells.

AGT RIA. AGT levels in cell culture medium and plasma from SHR were determined with a direct RIA. The antibody against rat AGT and purified rat AGT were kindly supplied by Dr. C. Sernia (University of Queensland, St. Lucie, Australia; see Ref. 35). Plasma or cell culture medium of the appropriate dilution was incubated with iodinated AGT and antibody at 4°C overnight, and the antibody-bound fraction was precipitated with the secondary antibody. The sensitivity of the assay was 0.3 ng/tube.

Liver toxicity measurements. Levels of two enzymes reflecting liver toxicity, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured in the plasma samples collected from SHR before and after injection of AS plasmid with or without liposome in the Chemistry Laboratory of Shands Hospital, University of Florida Health Science Center, by using an Auto-Biochemical Analyzer.

Statistical analysis. Data were analyzed using standard statistical methods. Statistical analysis was performed by one-way ANOVA for AGT levels in the medium and two-way ANOVA for repeated measurements followed by the Student-Newman-Keuls test for individual comparisons. Group data are presented as means ± SE. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Expression of GFP in cultured cells. To test the transfection efficiency of pAAV-AGT-AS and pAAV-AGT-S in the rat hepatoma cell line H4-II-E, fluorescence of GFP was observed and photographed 48 h after transfection. Cells were counted under a microscope. Cells treated with only Lipofectamine were used as the control. The results showed that the efficiency of transfection was ~50% (Fig. 2).

Time course of GFP and AGT AS mRNA expression in transfected cells by RT-PCR. Expression of GFP gene and AGT-AS in the transfected cells was confirmed by RT-PCR of RNA extracted from hepatoma H4 cells transfected with pAAV-AGT-AS. The expression was continuous from 2 to 72 h after transfection (Fig. 3). Both transgenes were detectable starting at the 2-h time point and were present until at least 72 h (Fig. 3, A and B). No visible band was present in control cells treated with lipofectamine.

AGT level in the medium. After the transfection (48 h), the level of AGT in the sense plasmid-transfected cells was 303.8 ± 23.2 ng/mg protein (n = 20). This is an increase of 38.3% compared with Lipofectamine-only treated cells (219.6 ± 16.9 ng/mg, n = 7, P < 0.05). In the medium of cell cultures treated with pAAV-AGT-AS, the level was 114.5 ± 16.7 ng/mg (n = 20). This is a decrease of 47.9% compared with the control (P < 0.01), as shown in Fig. 4.

Expression of pAAV-AGT-AS in vivo. RT-PCR of RNA isolated from liver and heart of the rats injected in the tongue vein with 3 mg/kg of pAAV-AGT-AS showed expression of the AGT-AS vector in the heart and liver 3 days after injection. Seven days after injection, expression was detected in the liver only (Fig. 5).

BP reduction by naked AS plasmid. The effect of naked pAAV-AGT-AS DNA on the BP of adult SHR was monitored daily from 1 to 9 days after intravenous injection. The results showed that all three different doses of AS plasmid DNA produced a significant reduction in SBP compared with the control rats injected with saline or sense plasmid DNA. The hypertensive effect was dose dependent. AS plasmid DNA decreased BP by 12 ± 3.0 mmHg (0.6 mg/kg), 16.5 ± 2.2 mmHg (1.5 mg/kg), and 22.5 ± 5.2 mmHg (3 mg/kg). Increasing doses not only produced a larger drop in BP but also extended the length of time that BP was significantly lower. The lowest dose (0.6 mg/kg) reduced BP for only 2 days. The dose of 1.5 mg/kg reduced BP for 4 days,
whereas the dose of 3 mg/kg decreased BP for 6 days. The sense plasmid DNA did not cause any significant change in BP compared with the saline control group (Fig. 6).

Comparison of hypotensive effects between injection of plasmid alone and plasmid with liposomes. Figure 7 shows the difference in the effectiveness of decreasing BP between pAAV-AGT-AS injection with and without liposomes. BP declined significantly from day 1 to day 6 after injection of pAAV-AGT-AS alone compared with that in the control SHR injected with liposomes only or sense plasmid plus liposomes. The injection of the AS plasmid complexed with liposomes prolonged the decrease to 8 days. In addition, the decrease of BP in the AS plus liposomes group was more profound than that in the AS-alone group. There was a significant difference in BP on days 4–8 (P < 0.05) between these two groups.

AGT levels in plasma of SHR. AGT levels were detected in plasma collected on days 3, 5, and 7 after injection. It was shown that the decrease in plasma AGT level was consistent with the change in BP (Fig. 8). Liposome-encapsulated AS plasmid injection caused a more profound and longer decrease in plasma AGT level than naked plasmid injection (Fig. 9).

Liver toxicity. There were no obvious changes in the levels of plasma ALT and AST in SHR injected with pAAV-AGT-AS mixed with liposomes or pAAV-AGT-AS alone compared with the baseline values or SHR injected with saline (Table 1).

**DISCUSSION**

AGT, the precursor of the blood vessel constrictor peptide, ANG II, is an important determinant of BP and homeostasis (15, 18, 31). The observation that plasma AGT correlates with BP in humans suggests that circulating AGT plays an important role in the pathogenesis of hypertension (15). Recent studies using AS oligodeoxynucleotides (ODN), including the work from our laboratory, suggest that AGT may be one of the candidate genes for gene therapy of hypertension (12,
Even though plasma ANG II levels are not greatly elevated in SHR, the tissue RAS may contribute to the development and sustaining of hypertension. Therefore, AGT, as a pivotal substrate in the RAS, tissue or circulating, could be a target gene for treating high BP. AS strategy is attractive because it specifically blocks the transcription or translation of the target gene (13). For delivery of AS, in the present study, we chose a plasmid vector containing AAV ITRs. Plasmids containing AAV ITRs have unique properties that make them suitable for a variety of gene therapy applications (17). ITRs may promote efficient gene transfer from the cytoplasm to the nucleus or increase the stability of plasmid DNA and enable longer-lasting gene expression (1, 3, 8). AAV-based plasmids have been proven to drive strong and long gene expression in the in vitro studies (9, 28, 32, 39, 40). In the in vivo study, direct injection of AAV-based plasmids into skeletal muscles has brought a high-level transgene expression (2). AAV plasmids carrying interleukin-2 gene have been used successfully to modify tumor cells and immunize tumor-bearing animals. Reductions in metastatic tumor burden have been observed in breast, ovarian, lung, and prostate cancer animal models (6, 17, 37). The goal of this study was to test the hypotheses that AAV-based plasmid with AGT AS will cause a significant decrease in AGT levels both in vitro and in vivo, resulting in a significant decrease in BP in SHR.

The in vitro study showed that our AAV-derived constructs, both sense and AS, can efficiently transfect the rat hepatoma cell line H4-II-E. In our previous studies using AAV-based plasmid and other cell lines like NG108–15, ATt-20, and L929, we observed similar high efficiency of transfection (23). The transgenes were expressed in these cells as early as 2 h after transfection. The AS plasmid significantly decreased the secreted AGT level by 47.9% compared with the nonplasmid control. This result, together with the decrease in the level of plasma AGT in SHR, indicates that the synthesis of the candidate target gene product...
was successfully inhibited by the AS gene. Although the protein was reduced by AS inhibition, the AGT mRNA level did not show significant changes by semiquantitative RT-PCR (data not shown). This phenomenon has also been observed in other AS inhibition systems (13, 38). It appears that reducing target mRNA levels by stimulating RNase H is not necessary for inhibiting protein production. The AS mRNA can inhibit translation by hybridizing to the target mRNA and can prevent either ribosomal assembly or ribosomal sliding along the mRNA, without degrading or cleaving mRNA upon AS treatment. The sense plasmid caused an increase of 38.3% in the secreted level of AGT in the cell culture medium, but it did not produce any significant increase in the plasma AGT level. One explanation is that the basal RAS may be overexpressed in SHR; therefore, the increase of the AGT level by the sense plasmid is not enough to reach a significant detectable level in the plasma. The AGT level in local tissues should be increased theoretically, but this is more difficult to measure. Another explanation could be due to the physiological feedback mechanism. Even though the AGT level might be increased initially after the injection, it could be brought down to the baseline level by internal regulatory mechanisms or degraded in the plasma.

In the in vivo study, the results showed that a single intravenous injection of different doses of naked plasmid caused a significant reduction in BP in SHR. A dose-dependent hypotensive result was achieved, with consistent decreases in plasma AGT levels. These data support the concept that peripheral AGT plays an important role in the pathogenesis and maintenance of hypertension. AGT is predominately produced in the liver and released into the blood. The RT-PCR detection of AS AGT mRNA in SHR showed that the AS plasmid was expressed in the liver and lasted longer in the liver than in other tissues. These results suggest that the AS to AGT delivered in AAV-based plasmid vector by intravenous injection acts mainly in the liver where it inhibits the production of AGT by the parenchymal cells. Because the major source of circulating AGT was inhibited, this led to the decrease in BP. Although the brain RAS has been shown to contribute to BP (12), it is generally accepted that the blood-brain barrier is only permeable to very small lipophilic molecules (27). Both naked hydrophilic plasmid DNA and the DNA-liposome complex have been proven incapable to cross the blood-brain barrier after peripheral administration (14, 21, 26). Therefore, even though we did not measure AS-AGT-mRNA in the brain, we have reason to believe that our AS had very limited access to brain, if any, and it did not function in the brain in this case.

Liposomes have been used to encapsulate and deliver a variety of materials such as DNA and viral particles into cells. They are nontoxic, as we show in this study. When Tomita et al. (36) injected AS ODN against AGT mRNA encapsulated in liposomes combined with the agglutinins of the Sendai virus into the portal vein of SHRs, BP decreased transiently from day 1 to day 4 by up to 18 mmHg. However, they did not compare their results with naked AS ODN. Wielbo et al. (41) observed a decrease in BP of SHRs 24 h after injection of the AS ODN to AGT mixed with liposomes, whereas samples without liposomes were not effective. In the present study, both naked and liposome-encapsulated plasmid DNA caused a significant decrease in BP and in plasma AGT levels. They produced a reduction in BP for 6 and 8 days, respectively, when delivered at the dose of 3 mg/kg, with the BP decrease by up to 22.5 and 24.2 mmHg. This was 2 or 4 days longer and greater than AS ODN (36) injected at an optimized dose. Therefore, the plasmid enhances and extends the effectiveness of AGT AS. An interpretation of the relatively better effects of our experiments, even with naked plasmid DNA, compared with liposome-encapsulated AS ODNs might be that AAV ITRs facilitate the entry of plasmid DNA into the cells and enhance plasmid maintenance in the cells, thereby allowing mRNA production of the transgenes for a longer period of time (7, 17, 28, 37). The observation that plasmid encapsulated by liposomes caused a more profound and longer decrease in both BP and plasma AGT levels in SHR indicates that liposomes allow higher transfer efficiency of AAV-based plasmid DNA. This in vivo observation confirms and extends the previous in vitro observations reported by Philip et al. (28) and Fan et al. (7).

Although our AS plasmid caused a significant decrease in BP and had a longer hypotensive effect than AGT-AS-ODN, it did not normalize the BP. The decrease in BP did not correlate in percentage to the decrease in the level of plasma AGT. These results are similar to those observed by others who used AGT-AS-ODN (12, 36). Hypertension is a polygenic disease, and the mechanism of high BP in SHR is still not well known. Based on the above observations, it may be assumed that the BP in SHR is influenced by not only AGT but some other diverse factors as well.

One of the major challenges for a gene therapy approach to hypertension is the problem of delivery. Both AS ODN and viral vector approaches are being studied (29, 30). rAAV is particularly attractive for gene transfer because of its integration to the host cellular chromosome and high efficiency transduction of numerous cell types both in vivo and ex vivo. However, the package size limit of AAV particles still limits the use of this gene delivery system. The AAV plasmid vectors are not subjected to the package size limit of rAAV and are simple to make and use. These advantages, along with the potential enhancement and persistence of transgene expression promoted by ITRs compared with other nonviral gene transfer systems such as ODNs and other plasmids, make them useful as alternatives for gene delivery, particularly for the delivery of the genes with the size beyond the package capacity of an rAAV. However, as there is no compelling evidence that plasmids containing AAV ITRs integrate (17), it is hard for them to overcome their transient nature in vivo, like the transient hypotensive results observed in our study. So, for longer AS effects, AS gene, with the size
within the package capacity of an rAAV vector, delivered in an AAV vector is still recommended.

In summary, the results in this study show that systemic delivery of AS directed to AGT mRNA by an AAV-based plasmid vector is efficiently taken up and expressed, with a significant inhibition of AGT protein production and a concomitant decrease of BP. This occurs with or without liposomes, but the results are better with liposomes. We conclude that AS inhibition of AGT is a viable strategy for reducing hypertension that involves RAS activity. The effective delivery of AS by a plasmid vector based on AAV suggests that further studies with the plasmid packaged in an rAAV vector for a longer-lasting antihypertensive effect are warranted.

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Address for reprint requests and other correspondence: M. I. Phillips, Dept. of Physiology, College of Medicine, Box 100274, Univ. of Florida, Gainesville, FL 32610 (E-mail: MIP@phys.med.ufl.edu).

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