Original Research

Central Plasmid Antisense Administration Reduces Blood Pressure Inhibiting α_{2B} Adrenoceptor Gene Expression in Spontaneously Hypertensive Rats In Vivo

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Introduction: The involvement of central α_{2B} adrenoceptors (AR) in the maintenance of hypertension has been proven by a series of previous experiments, at least in a particular model of nephrogenic salt-induced hypertension. The aim of the present study was to investigate further the role of central α_{2B} AR in hypertension by applying antisense technology in another experimental model, the spontaneously hypertensive rat (SHR).
Methods: Plasmid antisense DNA against the α_{2B} gene was given by intracerebroventricular injection to salt-fed SHRs, while a control group received plasmid alone.
Results: There was a significant fall in blood pressure, by an average of 31 ± 12 mmHg, within the first twenty hours after injection in the antisense group. On the first post-injection day the blood pressure fell from 204 ± 5.3 mmHg to 176.8 ± 2.9 mmHg ($p=0.02$). However, no significant changes in blood pressure were noticed in the plasmid group. Body-weight in both groups remained stable during the experiment. A study of frozen brain sections of SHRs after antisense DNA injection suggested that the nucleus tractus solitarii was one of the expression sites, while there was no histological evidence of tissue disruption.
Conclusion: Central injection of antisense DNA targeting α_{2B} mRNA in the genetic model of hypertension of the SHR seems to have a significant hypotensive effect, at least on the first day of injection. The nucleus tractus solitarii seems to be the primary area of action of central α_{2B} AR in SHRs.

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Hypertension has been characterized as a major risk factor in many cardiovascular pathophysiological states, such as arteriosclerosis, stroke, coronary artery disease, heart failure, peripheral arterial disease and renal failure. The sympathetic nervous system and its adrenergic central and peripheral receptors play a significant pathogenic role in the development and maintenance of hypertension. Although the stimulation of α_1 adrenoceptors increases blood pressure, stimulating α_2 receptors may lead to either a hypertensive or an opposite hypotensive result, inhibiting normal norepinephrine release from neural synapses.[1]

The α_2 adrenoceptors (AR) are divided into three subtypes, α_{2A}, α_{2B} and α_{2C}. The role of α_{2B} AR in hypertension has been mostly clarified. However, our previous studies in genetically engineered mice have suggested that the central α_{2A} AR is the predominant subtype. Since subtotal nephrectomized mice lacking a full compliment of α_{2B} AR gene are unable to raise their blood pressure in response to chronic salt loading, the salt-induced hypertension in mice is mediated primarily by central α_{2B} receptors.[2-4]

Spontaneously hypertensive rats are a genetic model of hypertension that has been widely used in research. An interesting fea-

ture of these animals is that they manifest much of the pathology normally seen in human hypertension.[5-6]

Since hypertension is a lifelong disorder and its treatment includes major limitations, such as poor compliance, side effects and short duration, novel therapeutic strategies must be explored. Gene therapy based on the over-expression or inhibition of specific genes is a new approach to hypertension treatment. Specificity and longer-lasting effects give gene therapy an attractive advantage over conventional drug treatment. Antisense strategy, through a number of mechanisms, offers the exciting possibility of inhibiting the expression of a particular gene without any changes in the functions of other genes.[7] As a result, only the expression of the target proteins is down-regulated. It is believed that a genetic approach to hypertension therapy, using sense or antisense technology, will be the treatment of choice in the future.[8]

Previous hypertension studies in our laboratory, using antisense oligodeoxynucleotide technology against rat α_{2B} mRNA in the central nervous system, in salt-induced hypertension, presented hypotensive results for several hours.[9] Moreover, after central injection of antisense DNA delivered via plasmid vector, we managed to reduce salt-induced hypertension for several days.[10] Both the above findings are indicative of the significant role of a fully functioning central α_{2B} AR in salt-induced hypertension as well as of the potential of gene therapy treatment.

In order to investigate further the role of central α_{2B} AR in hypertension, we administered intracerebroventricular plasmid antisense against α_{2B} AR, using another experimental model of hypertension, the spontaneously hypertensive rat.

Material and methods

The construction and production of the plasmid as well as the *in vivo* transfection efficiency of pAd-Track-CMV-α_{2B}-AR-antisense in NG108-15 cells through inhibition of protein expression have already been described in previous studies.[9]

Experimental model

Seventeen male spontaneously hypertensive rats, 11-19 weeks old and weighing 245-325 g, were used in these experiments, which were conducted in accordance with the guidelines for the Care and Use of Animals approved by the Boston University Medical Center.

Under general anesthesia with pentobarbital (50 mg/kg intraperitoneally), a PA-C40 radio-telemetry blood pressure transmitter probe (Data Sciences International, St. Paul, MN) was implanted in the aorta of each rat. One centimeter of the distal end of the catheter (0.7 mm diameter) was inserted upstream into the abdominal aorta above the bifurcation and fixed in position by placing two drops of medical-grade tissue adhesive and a 2 mm^2 cellulose fiber patch at the vessel entry site. The body of the transmitter was sutured to the abdominal wall. The same day a 24 gauge guide cannula (Plastic One, Roanoke, VA) was implanted stereotaxically into the left lateral ventricle of the brain, according to the following coordinates: 0.8 mm posterior of bregma, 1.3 mm lateral to the midline and 3.5 mm below the skull surface. The cannula was anchored to the skull with 3 screws and cranioplastic cement.

Five to seven days later the animals were fully recovered, baseline blood pressure and heart rate measurements were taken for three days and finally the rats were placed on an 8% NaCl diet for a period of three weeks. Data were stored using a computer-based radiotelemetry transmitter/receiver system (Data Sciences International, St. Paul, MN). The radiotelemetry system allows rats to be studied in regular cages without anesthesia or restraints that could influence blood pressure or heart rate results.

Intracerebroventricular delivery of plasmid DNA

On the day of either antisense-plasmid or plasmid injection, a 31-gauge inner cannula (Plastic One, Roanoke, VA) was lowered into the guide cannula, projecting one mm below the end (4.5 mm) and attached to a Harvard infusion pump (model PHD 2000, Harvard Apparatus, Holliston, MA). Injections were made with a 25 μl Hamilton syringe.

We injected 500 μg of pAd-Track-CMV-α_{2B}-AR-antisense in eight spontaneously hypertensive rats (AS group, n=8) or pAd-Track-CMV in nine animals (PL group, n=9) in 20 μl of 150 mmol/l sodium phosphate buffer at the rate of 0.125 μl/min for over 2 hours. During this procedure the rats were anesthetized and moving freely in their cage.

Blood pressure and heart rate were continuously measured for 30-second periods at 2-minute intervals for the entire 7-day experimental period. At the end of each experiment the location of the cannula within the lateral ventricle and the integrity of the surrounding tissues were confirmed histologically.

Expression of green fluorescein protein after intracerebroventricular injection of antisense

At the end of the experiment three rats were additionally infused with 500μg of pAd-Track-CMV-α_{2B}-AR-antisense over a 2-2.5h period in the left lateral brain ventricle. After 72h the animals were anesthetized and euthanized. Frozen brains were cryostat-sectioned into 30 μm thick sections. Distribution of green fluorescein protein (GFP) in the brain was ascertained by viewing with a fluorescein microscope.

Statistical analysis

Data are presented as mean ± SEM. Differences within and between groups were calculated using the paired and unpaired Student t-test, respectively. Differences at $p<0.05$ were considered significant.

Results

Spontaneously hypertensive rats are genetically hypertensive animals that develop hypertension in adult life. Indeed, our rats presented elevated values of systolic blood pressure at the beginning of the experiment, which were further augmented after salt loading (AS group, baseline 168.7 ± 5.3 mmHg vs. salt loading 203.7 ± 9 mmHg, $p<0.01$ and PL group, baseline 164 ± 3.9 mmHg vs. salt loading 195.1 ± 6 mmHg, $p=0.001$) (Figure 1).

After intracerebroventricular injection, all animals were monitored for seven days with respect to systolic blood pressure along with the heart rate. The AS group revealed a significant fall in blood pressure by an average of 31 ± 12 mmHg within the first twenty hours after injection. The level of blood pressure for the next three days post-injection was 176.8 ± 2.9 mmHg ($p=0.02$), 189.7 ± 2.9 mmHg (p: NS) and 197.3 ± 4.8 mm Hg (p: NS) on days 1, 2 and 3, respectively. Subsequently, the blood pressure tended to recover gradually, reaching the pre-injection levels by the fourth day (206.1 ± 7.3 mmHg), while it was further but not significantly increased by the seventh day (215.1 ± 9.9 mmHg).

In contrast, the PL group showed a non-significant fall in blood pressure during the first three days post-injection, which turned to a gradual non-significant increase by the fourth day.

A non-significant increase in heart rate, observed in the AS group on the first day post-injection, was fully restored during the second day. However, in the PL group, heart rate increased from 313 ± 5.8 bpm measured on the injection day to 342.2 ± 5.4 bpm during the first day post-injection ($p<0.01$) and then returned to pre-injection levels.

Regarding body weight, both groups of animals presented a slight non-significant decrease, which remained stable during the experiment. Neither group of animals had any evidence of neurotoxicity.

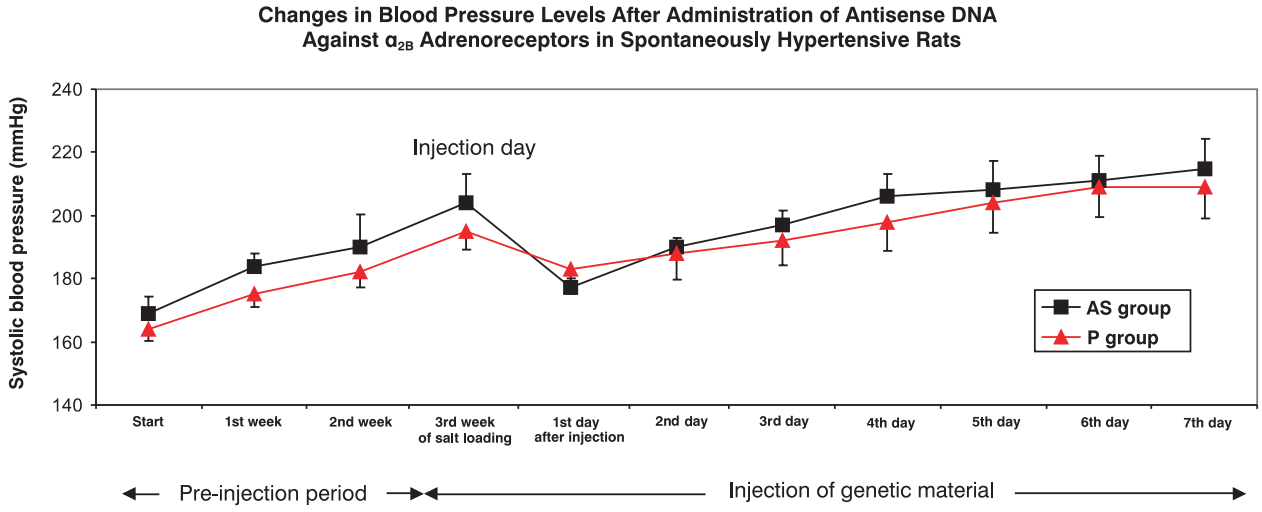

Figure 1. Blood pressure (BP) effect of intracerebroventricular delivery of antisense (AS) or plasmid (P) against α_{2B} gene in salt-fed spontaneously hypertensive rats. Systolic BP was elevated before the experiment and increased significantly during the next three weeks of dietary salt loading. The AS injection produced an immediate fall in BP, maximal at 20 h, which returned gradually to pre-injection levels during the 7 following days of observation. The plasmid group revealed no significant changes but a gradual continuing rise of BP over this period. *$p<0.05$ versus pre-injection BP levels.

After studying frozen brain sections of spontaneously hypertensive rats as for GFP expression after intracerebroventricular injection of antisense plasmid DNA, we concluded that the expression was clustered in isolated regions rather than being uniformly distributed throughout the brain section. This indicates that the uptake of the antisense plasmid DNA by the brain may be limited to regions that are accessible to direct intracerebroventricular injection. Fluorescent signal was detected in the brain areas adjacent to the third and fourth cerebral ventricles. The highest concentrations of the fluorescent signal were noted in the nucleus tractus solitarii.

Discussion

Adrenergic receptors, and subtypes, α_{2A}, α_{2B}, and α_{2C} in particular, appear to participate in blood pressure homeostasis by acting through the sympathetic nervous system. Through a sympathoexcitatory response α_{2B} adrenergic receptors are involved in the central regulation of vascular tone.[14]

Reviewing a series of experiments conducted in genetically engineered mice, we concluded that subtotally nephrectomized mice, lacking the α_{2B} gene and subjected to chronic dietary salt-loading, presented a diminished hypertensive response compared to animals with α_{2A} or α_{2C} gene knockout.[3,15-20]

Recently, using antisense technology in our laboratory, we suggested that translational inhibition of the central α_{2B} gene by antisense oligodeoxynucleotides (AS-ODNs) led to prevention of the expected blood pressure rise in an experimental model of salt-induced hypertension.[9] Subsequently, when instead of AS-ODNs we used antisense DNA delivered via plasmid vector in order to inhibit the translation of the α_{2B} gene in the central nervous system, we managed to partly reverse salt-induced hypertension for several days.[10]

The involvement of central α_{2B} AR in the maintenance of hypertension has been proven by that series of experiments, at least in a particular model of nephrogenic salt-induced hypertension.[21] Hypertensive animal models are essential in the process of development of new practical therapeutic methods based on gene transfer. Spontaneously hypertensive rats have been used in several previous studies where sense or antisense technology has been applied. Since essential hypertension appears to be a polygenic disease, several genes are likely to be involved in the genetic determination of blood pressure. While conventional therapy requires daily drug administration, gene therapy delivered by viral vectors, plasmid or naked DNA seems to have a long-lasting effect.[22]

The current study was designed to investigate the antihypertensive role of central inhibition of the α_{2B} gene in another model of hypertension, the spontaneously hypertensive rat, using antisense technology. For this purpose, a plasmid vector was chosen as a vehicle for antisense DNA, also containing a strong cytomegalovirus promoter for gene expression.

Gene therapy offers the possibility of producing long-lasting effects with precise specificity from the genetic design. We are currently using two strategies: AS-ODNs and antisense DNA delivered in viral vectors against genes with vasoconstrictive properties.[23-25] Antisense technology offers the possibility of inhibiting the expression of a particular gene, through mRNA production, without any changes in the functions of other genes.[26] Clinical trials using antisense in targeting AIDS, cancer and other genetic and acquired diseases indicate their potential clinical usefulness.[27]

One of the major challenges for a gene therapy approach to hypertension is the problem of delivery. Plasmids seem to be effective and safe vectors, as they do not integrate into the genome. As for their preparation, they are simple to make and use and they are not subject to a package limit since they do not require the more complex packaging needed for recombinant adeno-associated virus. Their effect lasts for a shorter time compared with viral vectors, producing blood pressure reduction lasting about one week.[28-29] Viral vectors used for gene delivery, although more effective, may have an opportunity for chromosomal insertion and therefore chromosomal breakage or carcinogenesis.[22] Therefore, delivery of viral vectors needs more engineering to ensure safety, but one day they may be used for the long-term control of blood pressure. Until then AS-ODNs will probably be developed first, because they can be treated as drugs for the treatment of hypertension with long-term effects.[23-25]

In our study, we chose the vector pAdTrak-CMV, which is used for production of GFP-trackable adenoviruses containing transgenes under the control of a strong promoter.[12] The same vector was chosen when we managed to significantly reduce blood pressure in subtotally nephrectomized, salt-fed rats with antisense plasmid DNA against the α_{2B} gene, following significant decrease of α_{2B} protein levels *in vitro* and *in vivo,* as previously described.[10]

Previous results of gene therapy for hypertension in spontaneously hypertensive rats have demonstrated sustained reduction and slowing of hypertension de-

velopment, as well as of left ventricular hypertrophy, with a single dose administration of antisense DNA against the angiotensinogen or AT1R gene. Recently, B_1 receptors, calcium receptors and other components of the renin angiotensin system, along with the kallikrein gene, have been used as targets of either antisense or sense gene therapy.[25] Indeed, direct gene delivery of human tissue kallikrein caused a sustained reduction in systolic blood pressure in spontaneously hypertensive rats, lasting for six weeks.[22] Administration of β1-AS-ODN in spontaneously hypertensive rats resulted in a profound and prolonged reduction in blood pressure for about three weeks without affecting heart rate.[27]

However, remarkable results were obtained when the renin angiotensin system was the target of gene therapy. The blood pressure reduction produced by systemic delivery of either AS-ODN or antisense DNA against the angiotensinogen gene in spontaneously hypertensive rats was sustained for a period from a few days to one week. When cationic liposomes were used the antihypertensive result was enhanced.[28,30] Virally mediated intracardiac gene delivery of antisense DNA against the angiotensin II type 1 receptor gene in spontaneously hypertensive rats resulted in a significant blood pressure reduction, maintained for up to 36 days, as well as reversed renovascular pathophysiology.[31] When antisense DNA against angiotensin converting enzyme was administered in spontaneously hypertensive rats, an attenuation of high blood pressure was observed, associated with the prevention of cardiac and renovascular pathophysiological alterations.[32]

In our experiment, adult, salt-fed, spontaneously hypertensive rats were given a single intracerebroventricular injection of plasmid antisense DNA against the α_{2B} gene. We were able to analyze the changes in blood pressure and heart rate in the conscious state, since anesthesia is known to influence cardiovascular parameters. We noticed a significant reduction in blood pressure by an average of up to 31 mmHg during the first twenty hours after injection. Subsequently, blood pressure remained significantly lower only the first post-injection day, while it then increased gradually and reached the pre-injection levels by the fourth day. Since no significant changes in blood pressure were noted when just plasmid DNA was injected, the specificity of the antihypertensive result of central administration of plasmid antisense DNA against the α_{2B} gene was proven. Additionally, GFP expression in areas adjacent to the cerebral ventricles, such as the nucleus tractus solitarii, indicates that plasmid DNA was taken up by those tissues. The nucleus tractus solitarii is known to be one of the sites of termination of primary afferent fibers from many cardiovascular receptors, such as α_{2B} AR.[33]

An interesting phenomenon was noticed in the plasmid group (control group) in terms of heart rate, which showed a significant increase only on the first post-injection day. Since no heart rate changes were observed in the plasmid antisense DNA group, we should consider that the experimental procedure itself might lead to a short duration reflex tachycardia through the sympathetic nervous system. This tachycardia was apparent only in the plasmid group, since antisense DNA injection resulted in sympathetic nervous system suppression through central α_{2B} AR.

No visual adverse effects of plasmid DNA delivery were noticed in our experiment, as judged by body weight, water or food intake, or any behavioral observations of the animals.

Our study confirms once again that α_{2B} AR play a significant role in the maintenance of hypertension, at least in the experimental model of salt-fed spontaneously hypertensive rats. Furthermore, the short reversal of severe hypertension in this model, taken together with our previous results where blood pressure remained significantly lower for a week,[10] leads us to believe that the primary role of α_{2B} AR is related to salt-induced hypertension.

Our results should be added to those regarding gene therapy and its future application in human hypertension. However, previous studies have shown that angiotensin converting enzyme antisense administration in spontaneously hypertensive rats was integrated into the genome and transmitted to the offspring.[32] Since the antihypertensive effect of gene therapy may not be appropriate in certain circumstances, such as risk of adverse effects, surgical procedures or pregnancy, the development of a regulated expression system on demand is necessary in order to modulate or turn off the expression of antisense, at least for a period of time. Those components of gene therapy should be resolved before the administration of the new therapeutic technology in human hypertension.

References

1. Aggarwal A, Esler MD, Socratous F, et al: Evidence for functional presynaptic alpha-2 adrenoceptors and their down-regulation in human heart failure. J Am Coll Cardiol 2001; 81: 1130.
2. Makaritsis PK, Johns C, Gavras I, et al: Role of the a2-adrenergic receptor subtypes in the acute hypertensive response to hypertonic saline infusion in anephric mice. Hypertension, 2000; 35: 609-613.

3. Makaritsis PK, Handy ED, Johns C, et al: Role of the a_{2B}-adrenergic receptor in the development of salt-induced hypertension. Hypertension, 1999; 33: 14-17.
4. Gavras I, Manolis AJ, Gavras H: The alpha$_2$-adrenergic receptors in hypertension and heart failure: experimental and clinical studies. J Hypertension 2001; 12: 2115-2124.
5. Lovenberg MW, Yamori Y: The role of dietary protein in hypertensive disease, in Laragh HJ, Brenner MB, (eds.): Hypertension. Pathophysiology, Diagnosis, and Management, vol. 1, 2nd edition. Raven Press, New York, 1995; p 314.
6. Okamoto K: Spontaneously hypertension in rats, in Richter GW, Epstein MA, (eds.): International review of experimental pathology, vol. 7. Academic Press, New York, 1969; pp 227-270.
7. Philips MI, Ambuhl P, Gyurko R: Antisense inhibition and adeno-associated viral vector delivery for reducing hypertension. Hypertension 1997; 29: 177-187.
8. Pachori SA, Huentelman JM, Francis CS, et al: The future of hypertension therapy; sense, antisense, or nonsense? Hypertension 2001; 37: 357-364.
9. Kintsurashvili E, Gavras I, Johns C, et al: Effects of antisense oligodeoxynucleotide targeting of the α_{2B}-adrenergic receptor mRNA in the central nervous system. Hypertension 2001; 38: 1075-1080.
10. Kintsurashvili E, Johns C, Ignjacev I, et al: Central [alpha] $_{2B}$-adrenergic receptor antisense in plasmid vector prolongs reversal of salt-dependent hypertension. J Hypertens 2003; 21: 961-967.
11. Zeng DW, Harrison JK, D'Angelo DD, et al: Molecular characterization of a rat [alpha] $_{2B}$-adrenergic receptor. Proc Natl Acad Sci USA 1990; 87: 3102-3106.
12. He TC, Zhou S, da Costa LT, et al: A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 1998; 95: 2509-2514.
13. Lowry OH, Rosenbrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
14. Bede M, Philipp M, Knaus A, et al: alpha2-adrenergic receptor subtypes novel functions uncovered in gene-targeted mouse models. Biol Cell 2004; 96(5): 343-348.
15. MacMilan LB, Hein L, Smith MS, et al: Central hypotensive effects of the of the a2a-adrenergic receptor subtype. Science 1996; 273: 801-803.
16. Link RE, Desai K, Hein L, et al: Cardiovascular regulation in mice lacking a_2-adrenergic receptor subtypes band c. Science 1996; 273: 803-805.
17. Link RE, Stevens MS, Kulatunga M, et al: Targeted inactivation of the gene encoding the mouse a_{2C}-adrenoceptor homolog. Mol Pharm 1995; 48: 48-55.
18. MacDonald E, Kobilka BK, Scheinin M: Gene targeting-homing in on a_2-adrenoceptor subtype function. Trends Pharm Sci 1997; 18: 211-219.
19. Altman JD, Trendelenburg AU, MacMilan L, et al: Abnormal regulation of the sympathetic nervous system in a_{2A}-adrenergic receptor knockout mice. Mol Pharm 1999; 56: 154-161.
20. Makaritsis PK, Johns C, Gavras I, et al: Sympathoinhibitory function of the a_{2A}-adrenergic receptor subtype. Hypertension 1999; 34: 403-407.
21. Feldman J, Bousquet P: Role of central a_{2B} adrenergic receptors in blood pressure control and hypertension. Journal of Hypertension 2003; 21: 871-872.
22. Wang C, Chao L, Chao J: Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. J Clin Invest 1995; 95: 1710-1716.
23. Philips MI: Gene therapy for hypertension: sense and antisense strategies. Expert Opin Biol Ther 2001; 1: 655-662.
24. Philips MI: Gene therapy for hypertension. The preclinical data. Methods Enzymol 2002; 346: 3-13.
25. Philips MI, Kimura B: Antisense therapeutics for hypertension: targeting the renin-angiotensin system. Methods Mol Med 2004; 106: 51-68.
26. Helene C, Toulme JJ: Specific regulation of gene expression by antisense, sense and antigene nucleic acids. Biochim Biophys Acta 1990; 1049: 99-125.
27. Zhang YC, Bui JD, Shen L, et al: Antisense inhibition of ß1-Adrenergic receptor mRNA in a single dose produces a profound and prolonged reduction in high blood pressure in spontaneously hypertensive rats. Circulation 2000; 101: 682-688.
28. Tang X, Mohuczy D, Zhang C, et al: Intravenous angiotensinogen antisense in AAV-based vector decreases hypertension. Heart Circ Physiol 1999; 46: H2392-H2399.
29. Philips MI: Gene therapy for hypertension. The preclinical data. Hypertension 2001; 38: 543-548.
30. Tomita N, Morishita R, Higaki J, et al: Transient decrease in high blood pressure by in vivo transfer of antisense oligodeoxynucleotides against rat angiotensinogen. Hypertension 1995; 26; 131-136.
31. Katovich MJ, Gelband CH, Reaves P, et al: Reversal of hypertension by angiotenensin II type 1 receptor antisense gene therapy in the adult SHR. Am J Physiol 1999; 277: H1260-H 1264.
32. Wang H, Reaves PY, Gardon ML, et al: Angiotensin I-converting enzyme antisense gene therapy causes permanent antihypertensive effects in the SHR. Hypertension 2000; 35: 202-208.
33. Hirooka Y, Sakai K, Kishi T, et al: Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. Hypertension Research 2003; 26: 325-331.