Effects of Antisense Oligodeoxynucleotide Targeting of the \( \alpha_{2B} \)-Adrenergic Receptor Messenger RNA in the Central Nervous System

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Abstract—The results of previous studies with genetically engineered mice have suggested that an intact central \( \alpha_{2B} \)-adrenergic receptor (\( \alpha_{2B} \)-AR) subtype mediates the development and maintenance of salt-induced hypertension. In the present study, we sought to further define the role of this receptor by injecting antisense oligodeoxynucleotides (AS-ODNs), targeting a selected sequence of the \( \alpha_{2B} \)-AR mRNA, into the lateral cerebral ventricle of rats that had undergone prior subtotal nephrectomy and dietary salt loading. Cell culture studies showed that these AS-ODNs could block \( \alpha_{2B} \)-AR protein generation. Before AS-ODN injection, blood pressure (BP) averaged 133 \( \pm \) 5 mm Hg during the daytime and rose to 165 \( \pm \) 4 mm Hg during the nighttime activity hours (\( P<0.001 \) versus baseline average of 120 \( \pm \) 2 mm Hg). The injection of AS-ODNs during the early afternoon prevented the BP rise and was associated with a significant fall in heart rate (from 385 \( \pm \) 12 to 306 \( \pm \) 15 bpm, \( P<0.05 \)) and symptoms of sedation that lasted for several hours, with a peak at 3 to 6 hours and full recovery by 24 hours. At that time, a second injection produced identical effects in all rats (\( n=9 \)). Control rats (\( n=10 \)) that received scrambled ODN injections had no changes in BP or heart rate patterns, and neither group had evidence of neurotoxicity, indicating that these effects are specifically due to translational inhibition of central \( \alpha_{2B} \)-AR. We conclude that a fully functional central \( \alpha_{2B} \)-AR is necessary for the induction of salt-dependent hypertension. (Hypertension. 2001;38:1075-1080.)

Key Words: hypertension, sodium dependent \( \bullet \) genes \( \bullet \) antihypertensive therapy

The \( \alpha_{2} \)-Adrenergic receptors (\( \alpha_{2} \)-ARs) are members of the superfamily of G protein–coupled receptors.\(^1\) There are 3 members of the \( \alpha_{2} \)-AR family: \( \alpha_{2A}, \alpha_{2B}, \) and \( \alpha_{2C} \) (on the basis of sequence homology, the \( \alpha_{2B} \) subtype is the rat equivalent to the human \( \alpha_{2C} \)-AR subtype).\(^2\) All are known to inhibit adenylate cyclase and to be involved in both central and peripheral control of blood pressure (BP), behavior, insulin release, sedation, and the presynaptic regulation of neurotransmitter release.\(^3-6\) \( \alpha_{2} \)-AR subtypes differ in their structure, patterns of tissue expression, and pharmacological profile.\(^7-11\)

Traditional pharmacological methods have a limited capacity to determine the functional role of receptor subtypes. In many cases, agents that display high selectivity within a family in vitro fail to maintain selectivity in vivo and can even interact with different classes of G protein–coupled receptors.\(^12,13\) Alternative approaches to defining the functional role of receptor subtypes include the generation of transgenic animals, which overexpress or lack the gene for a certain receptor or signaling component, and gene treatment. The latter involves amplification of a gene product via extra gene copies or inhibition of gene expression via antisense techniques.

Recently, genetically engineered mice with modified genes for any 1 of the \( \alpha_{2} \)-AR subtypes became available.\(^5,14\) This has already helped in the assignment of subtype-specific functions and should help in the design of new drugs. Studies with such animals have shown that the \( \alpha_{2A} \)-AR is primarily responsible for the centrally mediated and clinically beneficial hypotensive effect of \( \alpha_{2} \)-AR stimulation by agonists, such as clonidine, whereas the \( \alpha_{2C} \)-AR is the primary mediator of the hypertensive effects of \( \alpha_{2} \)-AR stimulation.\(^4,5\) Recent data from our laboratory have shown that the \( \alpha_{2B} \)-AR subtype is necessary in the hypertensive response to salt loading and suggested that the pressor effect is a function of \( \alpha_{2B} \)-AR located in the central nervous system (CNS).\(^15,16\) not in the periphery, as proposed by other investigators.\(^5\) To further assess the role of this subtype in salt-induced hypertension, we attempted to selectively suppress its expression in the CNS using antisense technology, which provides a highly specific and reversible means for the inhibition of protein expression. To this aim, we injected antisense oligodeoxynucleotides (AS-ODNs) targeted to rat \( \alpha_{2B} \)-AR mRNA into the lateral cerebral ventricle of rats with salt-induced hypertension under constant monitoring of BP. We hypothesized that inhibition of the generation of the \( \alpha_{2B} \)-AR would decrease BP in these animals.

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Methods

ODN Synthesis and Use

AS-ODNs were synthesized as phosphorothioated 18-mers targeted to bases 12 to 29 of the rat \( \alpha_{2B} \)-AR coding sequence according to the RING \( \alpha \) sequence of Zeng et al.\(^{17} \) The antisense sequence chosen was 5'-GCTCTGATGTTCTCAGTGTG-3'. As a control, we used scrambled ODNs, the sequence of which was 5'-TGACGCTCTCGCTGTGTA-3'. Fluorescein isothiocyanate (FITC)-conjugated ODNs were composed of phosphorothioated sequence with FITC conjugation at the 5' ends. The phosphorothioated AS-ODNs and scrambled ODNs were synthesized at Gemini Biotech. All oligomers were purified by HPLC.

In Vitro Testing of Rat \( \alpha_{2B} \)-AR Inhibition by AS-ODNs

The capacity of AS-ODNs to inhibit rat \( \alpha_{2B} \)-AR gene expression was tested on mouse neuroblastoma×rat glioma NG108-15 hybrid cells. This cell line is known to express \( \alpha_{2B} \)-AR.\(^{18} \) Reverse transcription–polymerase chain reaction (RT-PCR) was performed to ascertain rat-specific expression of the \( \alpha_{2B} \)-AR.

Uptake of AS-ODNs by cells expressing rat \( \alpha_{2B} \)-AR was shown by the addition of 2 \( \mu \)mol/L FITC-labeled AS-ODNs into the culture medium. After 24 hours of incubation, the cells were fixed and visualized using a fluorescent microscope.

The culture media with 2 \( \mu \)mol/L phosphorothioated AS-ODNs, scrambled ODNs, or PBS were changed twice a day for 3 days. After 72 hours, the NG108-15 cells were harvested, and membranes from cells were isolated as described by Phillips et al.\(^{19} \) Protein content of each experiment, the location of the cannula within the lateral ventricle and the integrity of surrounding tissues were confirmed for the entire 8-day period of the experiments. Behavioral changes in each rat were assessed on the basis of the righting reflex. At the end of each experiment, the location of the cannula within the lateral ventricle and the integrity of surrounding tissues were confirmed histologically.

Distribution of FITC-Conjugated AS-ODNs in the Brain

Next, 10 \( \mu \)L of 5 nmol FITC-conjugated AS-ODNs was infused over 15 minutes in the left lateral brain ventricle of rats (n=3). One hour later, the animals were anesthetized and killed. Frozen brain was cryostat-sectioned into 30-\( \mu \)m-thick sections and viewed with a microscope.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Detection of Rat \( \alpha_{2B} \)-AR Gene in NG108-15 Cells by RT-PCR and Cellular Uptake

The rat-specific expression of the \( \alpha_{2B} \)-AR gene in hybrid NG108-15 cells was ascertained by RT-PCR. With mouse-specific primers, no product was detected, whereas an expected 365-bp fragment was detected with primers recognizing both mouse and rat sequences, indicating that the NG108-15 cells express a rat \( \alpha_{2B} \)-AR transcript.

The uptake and intracellular localization of FITC-labeled AS-ODNs by NG108-15 cells after 24-hour incubation in culture were visualized using a fluorescent microscope.

Inhibition of Rat \( \alpha_{2B} \)-AR Gene in NG108-15 Cells In Vitro

After 3-day AS-ODN treatment of NG108-15 cells, there was a clear reduction in immunodetectable rat \( \alpha_{2B} \)-AR protein as shown by Western blot analysis with \( \alpha_{2B} \)-AR-specific antibodies. Thus, the rat \( \alpha_{2B} \)-AR protein level was significantly reduced in NG108-15 cells treated with rat \( \alpha_{2B} \)-AR AS-ODNs (by 52%, \( P<0.05 \)), compared with cells treated with the PBS control or scrambled ODNs, indicating the efficacy of the AS-ODNs for inhibition of \( \alpha_{2B} \)-AR expression.

Inhibition of Rat \( \alpha_{2B} \)-AR Gene In Vivo

The effect of intracerebroventricular AS-ODNs (n=9) or scrambled ODNs (n=10) on BP in rats that underwent subtotal nephrectomy and received 1% NaCl as drinking water is shown in Figure 1. After \( \geq \)3 days of baseline BP recording, the animals underwent 3 days of salt loading, during which systolic BP rose significantly in all animals during the nighttime activity hours, with lesser increases during daytime sleep hours.

The average 24-hour baseline BP for both groups was 120±2 mm Hg, with minimal daytime/nighttime variability. During the 3 days of salt loading, the nighttime BP was 163±4 mm Hg (\( P<0.001 \) from baseline), whereas daytime BP was 133±5 mm Hg (not significantly different from baseline). The injection of AS-ODNs in the early afternoon prevented the expected BP rise during the next several hours, with the greatest effect apparent at 3 and 6 hours postinjection, when the rats receiving scrambled ODNs were already exhibiting a marked BP elevation. By 24 hours, there was no difference between the rats that had received AS-ODNs and those that had received the scrambled ODNs. The second injection of AS-ODNs or scrambled ODNs was given at that time and produced essentially the same effect as the first one. Figure 2 shows detailed individual BP recordings of 1 AS-ODN-injected and 1 scrambled ODN-injected rat.

Heart rate was not altered by salt loading but significantly decreased after AS-ODN injections and returned to control level by 24 hours (388±12.9 bpm at baseline, 385±11.9 bpm during salt loading, and 306±15.4 bpm at 3 to 6 hours postinjection; \( P<0.05 \) versus both control and salt-loading periods). The second AS-ODN injection had a similar effect on heart rate as the first injection. Scrambled ODN injections caused no significant changes in heart rates (402±6.0,
405±10.5, 384±14.9, and 388±11.7 bpm at baseline, salt loading, 3 to 6 hours postinjection, and 24 hours postinjection, respectively).

**Behavioral Changes**

Within 30 minutes from the AS-ODN injection, the rats displayed signs of sedation (ie, they were unable to right themselves for several seconds after being placed on their back, had difficulty raising their head from the floor of the cage, and appeared to lose balance). These behaviors peaked at ≈4 hours and wore off gradually. By the next day, the rats had fully recovered and again responded in the same manner to the second AS-ODN injection. The scrambled ODNs produced no behavioral changes.

**FITC-Conjugated AS-ODN Distribution in the Brain**

To confirm the location of AS-ODNs delivered through the cannula, FITC-conjugated AS-ODNs were injected as described in Methods. Fluorescent signal was detected in the brain areas adjacent to the third and fourth cerebral ventricles. The highest concentrations of the fluorescent signal were found in the tissues bordering the ventricles and the cerebellum. High concentrations of fluorescent deposits were noted in the nucleus tractus solitarii, locus ceruleus, dorsal raphe nucleus, ventral parabrachial nucleus, and central gray pons. The optic nerve also displayed a strong fluorescent signal. Figure 3 shows representative brain sections.

**Discussion**

In this study, we demonstrated that when AS-ODNs targeted to a complementary sequence of rat α2B-AR mRNA are injected directly into the CNS of rats, they produce a significant attenuation of salt-induced hypertension accompanied by a fall in heart rate. The specificity of this effect is shown by the fact that scrambled ODNs administered into the third cerebral ventricle of subtotally nephrectomized, salt-fed hypertensive rats in the same manner produced no changes in the BP or heart rate patterns. The data indicate that translational inhibition of the central α2B-AR can prevent the salt-induced BP rise in this typical experimental model of salt-dependent hypertension.

These results are consistent with previous studies by other investigators, who have shown in genetically engineered animals that the α2B-AR has a hypertensive function. Furthermore, our recent studies showed that the induction of hypertension via short- or long-term salt loading requires a full complement of functional α2B-AR. We also presented evidence that this is a function of presynaptic centrally located α2B-AR. Indeed, the only peripheral postsynaptic α2-AR subtype capable of direct vasoconstriction in response to catecholamines (under α1-AR blockade) is α2A-AR, and α2B-AR mRNA could not be detected in arterial wall tissues via in situ hybridization, whereas it was abundantly present in CNS nuclei, along with mRNA for the other 2 α2-AR subtypes.

Experimental gene treatment of hypertension has been attempted via various methods in recent years. Approaches include the delivery of extra copies of candidate genes whose products contribute to vasodilation and the inhibition of expression of genes whose products contribute to vasoconstriction. Inhibition of gene expression can be achieved through the administration of AS-ODNs against a targeted mRNA sequence, either directly or via viral vectors. For the present study, we chose an AS-ODN
sequence that was located downstream of the initiation site, because the region that encompasses the codon AUG of rat \(\alpha_{2\beta}\)-AR mRNA includes 4 consecutive C nucleotides and the corresponding antisense sequence would contain a G quartet, which may cause nonspecific toxic effects.\(^{27}\)

The specificity and efficacy of the AS-ODNs against rat \(\alpha_{2\beta}\)-AR mRNA were confirmed in vitro on NG108-15 cells, which are known to express the rat \(\alpha_{2\beta}\)-AR subtype.\(^{28}\)

Indeed, we demonstrated that when AS-ODNs were added to the culture medium of these cells, they resulted in markedly decreased production of \(\alpha_{2\beta}\)-AR protein in vitro. Therefore, the attenuation of BP rise in vivo represents a specific effect of inhibition of the expression of these receptors in the CNS areas adjacent to the site of intracebroventricular injection, as shown by the fluorescent signal of AS-ODN distribution in those areas. Scrambled ODNs had no effect on \(\alpha_{2\beta}\)-AR protein production in vitro or the heart rate and BP pattern in vivo, arguing against a nonspecific effect. The lack of tissue damage around the ventricles suggests the absence of neurotoxicity.

Our results are at variance with those of Nunes,\(^{29}\) who found no BP effect with the central administration of AS-ODNs directed to a different region of the rat \(\alpha_{2\beta}\)-AR transcript. Because he found a hypertensive effect from AS-ODN inhibition of the \(\alpha_{2\lambda}\)-AR, he concluded that only the \(\alpha_{2\lambda}\)-AR subtype has a role on central BP regulation, a

Figure 2. Representative individual BP recordings during the entire experiment in 1 AS-ODN–treated rat (top) and 1 scrambled ODN–treated rat (bottom). BP is normal with minimal variability during the first 3 baseline days and starts increasing, with a large diurnal variation, after initiation of dietary salt loading with saline as drinking water. On days 6 and 7, the rats receive treatment with intracebroventricular injection. There is a delayed rise and lower peak BP in the AS-ODN–treated rat (top), whereas BP progressively increased in the control rat (bottom).
conclusion that is contrary not only to our findings but also to those of others who have used genetically engineered mice and found a hypertensive influence of \( \alpha_{2B}\)-AR.4,5

The magnitude of the antihypertensive action of our current approach is comparable to that achieved by other investigators who used AS-ODNs against other candidate genes. Examples include AS-ODNs directed against the mRNA of various components of the renin-angiotensin system, such as angiotensinogen, angiotensin II type 1 receptor, ACE, and the renin gene. The onset and duration of the BP-lowering effect vary and have been reported to occur within 3 to 9 hours and to last for 3 to 7 days.26 It is unclear at this point whether the time frame is determined mainly by the kinetic characteristics of the targeted proteins or whether the mode of delivery (eg, choice of vector, encapsulation in liposomes, and so on) can enhance the efficiency or prolong the half-life of AS-ODNs. In the present study, the onset of behavioral changes occurred at \( \approx 30 \) minutes after the injection of AS-ODNs and peaked at \( \approx 4 \) hours, whereas the attenuation of BP rise was most apparent between 3 and 6 hours postinjection and lasted <24 hours. These observations imply a rather rapid turnover of \( \alpha_{2B}\)-AR. The half-life of the \( \alpha_{2B}\)-AR has not yet been fully elucidated and varies widely in different reports, with the shortest being 1.2 hours,30 which is consistent with our findings. It is also possible that the turnover of \( \alpha_{2B}\)-AR differs among various tissues and even across areas of the CNS or that a more functionally active pool of receptors has a more rapid turnover rate.31

Interestingly, in addition to the antihypertensive effect, the CNS injection of AS-ODNs against \( \alpha_{2B}\)-AR mRNA produced symptoms of sedation and loss of equilibrium. The latter symptom is consistent with the fact that \( \alpha_{2B}\)-AR mRNA was found to be abundantly present in cerebellar structures.25 The sedation was unexpected, because the sedative, anesthetic, and sympatholytic effects of pharmacological nonselective \( \alpha_2\)-AR agonists have been generally attributed to the \( \alpha_{2A}\)-AR or \( \alpha_{2C}\)-AR subtype in studies with genetically engineered mice,32,33 and these results have been corroborated by the use of AS-ODNs against \( \alpha_{2A}\)-AR.34 \( \alpha_{2B}\)-AR is believed to have mainly a hypertensive role5,15,16 and a poorly defined developmental function,35 whereas \( \alpha_{2C}\)-AR has been implicated mostly in behavioral alterations.33,35 However, the present findings indicate that it is difficult to separate the hemodynamic from the behavioral aspects of sympathetic interventions. The use of pharmacological agents with central \( \alpha_2\)-AR agonistic properties, such as clonidine, for the treatment of hypertension is limited by side effects such as drowsiness that have been attributed to indiscriminate binding of these agents with all \( \alpha_2\)-AR subtypes. It is, however, possible that this difficulty is not due to nonselectivity of the drugs but that it may indicate that sympathetic suppression per se is inevitably accompanied by such symptoms.

In conclusion, our data indicate that the inhibition of central \( \alpha_{2B}\)-AR gene expression through the specific targeting of mRNA with AS-ODNs injected into the lateral cerebral ventricle can prevent for several hours the expected BP rise in subtotally nephrectomized rats that underwent dietary salt loading, thus further corroborating the notion that a fully functional central \( \alpha_{2B}\)-AR is a necessary mediator of salt-induced hypertension. The specificity of this effect is shown by the fact that scrambled ODNs have no influence on BP or heart rate patterns.
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References
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