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

Ekaterina Kintsurashvili, Irene Gavras, Conrado Johns, Haralambos Gavras

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±5 mm Hg during the daytime and rose to 165±4 mm Hg during the nighttime activity hours (\(P<0.001\) versus baseline average of 120±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±12 to 306±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 ■ genes ■ 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 \(\alpha_{2A}\)-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.\(^5\)–\(^5\),\(^14\) 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.

Received April 5, 2001; first decision April 16, 2001; revision accepted May 7, 2001.
From the Hypertension and Atherosclerosis Section, Department of Medicine, Boston University School of Medicine, Boston, Mass.
Correspondence to Haralambos Gavras, MD, Chief, Hypertension & Atherosclerosis Section, Boston University School of Medicine, 715 Albany St, Boston, MA 02118. E-mail hgavras@bu.edu
© 2001 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org

1075
Methods

ODN Synthesis and Use
AS-ODNs were synthesized as phosphorothioated 18-mer targets to bases 12 to 29 of the rat α2B-AR coding sequence according to the RING α sequence of Zeng et al. The antisense sequence chosen was 5′-CTCCTGATGGTCCATGGT-3′. As a control, we used scrambled ODNs, the sequence of which was 5′-TGACGTCTCCTCGGTGTA-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 α2-AR Inhibition by AS-ODNs
The capacity of AS-ODNs to inhibit rat α2B-AR gene expression was tested on mouse neuroblastoma×rat glioma NG108-15 hybrid cells. This cell line is known to express α2-AR,18 reverse transcription–polymerase chain reaction (RT-PCR) was performed to ascertain rat-specific expression of the α2B-AR.

Uptake of AS-ODNs by cells expressing rat α2B-AR was shown by the addition of 2 μmol/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 μmol/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 was assayed according to Lowry et al.19 Proteins were subjected to Western blot analysis for rat α2B-AR protein level assessment.

Animals and Procedures
Twenty-two male Wistar rats, each weighing 237 to 396 g (Charles River Laboratories Inc, Wilmington, Mass), 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.

For subtotal nephrectomy,21 rats first had both poles of the left kidney excised while under anesthesia. Five to 7 days later, a 24-gauge guide cannula (Plastic One) was implanted stereotaxically into the left lateral ventricle. Five to 7 days after that, a PA-C40 radiotelemetry BP transmitter probe (Data Sciences International) was implanted in the aorta. Five to 7 days later, rats had the right kidney removed. After baseline measurements were taken for 5 days, rats were placed on 1% NaCl as drinking water.

On the day of the AS-ODN injection, a 31-gauge inner cannula (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump. Injections (Plastic One) was lowered into the guide cannula, projecting 1 mm below the end (4.5 mm), and attached to a Harvard pump.

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 rat 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, 406±6.0 bpm at baseline and 388±11.9, 385±11.9, and 305±15.3 at 3, 6, and 24 hours postinjection, respectively; P>0.05).

Results

Detection of Rat α2B-AR Gene in NG108-15 Cells by RT-PCR and Cellular Uptake
The rat-specific expression of the α2B-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 α2B-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 α2B-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 α2B-AR protein as shown by Western blot analysis with α2B-AR–specific antibodies. Thus, the rat α2B-AR protein level was significantly reduced in NG108-15 cells treated with rat α2B-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 α2B-AR expression.

Inhibition of Rat α2-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 ≥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.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.
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.4,5 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.15,16 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,22 and α3B-AR mRNA could not be detected in arterial wall tissues via in situ hybridization,23,24 whereas it was abundantly present in CNS nuclei, along with mRNA for the other 2 α2-AR subtypes.25

Experimental gene treatment of hypertension has been attempted via various methods in recent years.26 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 α3β-AR mRNA includes 4 consecutive C nucleotides and the corresponding antisense sequence would contain a G quartet, which may cause nonspecific toxic effects. The specificity and efficacy of the AS-ODNs against rat α3β-AR mRNA were confirmed in vitro on NG108-15 cells, which are known to express the rat α3β-AR subtype. Indeed, we demonstrated that when AS-ODNs were added to the culture medium of these cells, they resulted in markedly decreased production of α3β-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 α3β-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, who found no BP effect with the central administration of AS-ODNs directed to a different region of the rat α3β-AR transcript. Because he found a hypertensive effect from AS-ODN inhibition of the α2A-AR, he concluded that only the α2A-AR subtype has a role on central BP regulation, a
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 role 5, 15, 16 and a poorly defined developmental function, 35 whereas $\alpha_2A$-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 subtotaly 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.
Acknowledgment
This work was supported by National Heart, Lung, and Blood Institute grant P50-HL-55001.

References
Effects of Antisense Oligodeoxynucleotide Targeting of the α2B-Adrenergic Receptor
Messenger RNA in the Central Nervous System
Ekaterina Kintsurashvili, Irene Gavras, Conrado Johns and Haralambos Gavras

_Hypertension_, 2001;38:1075-1080
doi: 10.1161/hy1101.093426

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/38/5/1075

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2001/11/06/38.5.1075.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/