Antisense Inhibition of $\beta_1$-Adrenergic Receptor mRNA in a Single Dose Produces a Profound and Prolonged Reduction in High Blood Pressure in Spontaneously Hypertensive Rats

Yuan Clare Zhang, BS; Jonathan D. Bui, PhD; Leping Shen, BS; M. Ian Phillips, PhD, DSc

**Background**—$\beta$-Blockers are the first line of therapy for hypertension. However, they are associated with side effects because of central nervous system (CNS) effects and $\beta_2$-adrenergic antagonism. To overcome these problems and provide a long-term $\beta_1$-blockade, antisense oligonucleotides against rat $\beta_1$-adrenergic receptor ($\beta_1$-AR) mRNA ($\beta_1$-AS-ODN) were designed and tested for the ability to inhibit cardiac $\beta_1$-ARs as well as lower blood pressure in spontaneously hypertensive rats (SHRs).

**Methods and Results**—Radioligand binding assay showed that a single intravenous injection of $\beta_1$-AS-ODN delivered in cationic liposomes significantly decreased cardiac $\beta_1$-AR density by 30% to 50% for 18 days ($P<0.01$), with no effect on $\beta_2$-ARs. This was accompanied by marked attenuation of $\beta_1$-AR–mediated positive inotropic response in isolated perfused hearts in vitro ($P<0.02$) and in conscious SHRs monitored by telemetry in vivo ($P<0.02$). Furthermore, the blood pressure of SHRs was reduced for 20 days, with a 38 mm Hg maximum drop. Heart rate was not significantly decreased. Quantitative autoradiography was performed to assess $\beta_1$-AS-ODN effects on the CNS, which demonstrated no changes in $\beta_1$-ARs in brain, in contrast to a significant reduction in heart and kidney ($P<0.05$). For comparison with $\beta$-blockers, the effects of atenolol on cardiovascular hemodynamics were examined, which lowered blood pressure for only 10 hours and elicited appreciable bradycardia in SHRs.

**Conclusions**—These results indicate that $\beta_1$-AS-ODN, a novel approach to specific $\beta_1$-blockade, has advantages over currently used $\beta$-blockers in providing a profound and prolonged reduction in blood pressure without affecting heart rate, $\beta_1$-ARs, and the CNS. Diminished cardiac contractility resulting from less $\beta_1$-AR expression contributes to the antihypertensive effect. (*Circulation. 2000;101:682-688.*)

**Key Words:** gene therapy $\square$ receptors, adrenergic, beta $\square$ hypertension $\square$ contractility

The sympathetic nervous system plays a crucial role in the regulation of blood pressure (BP), mainly through activating $\alpha$- and $\beta$-adrenergic receptors ($\beta_2$-ARs) in the effector organs, including heart, kidney, and blood vessels. Adrenergic-blocking agents, especially $\beta$-blockers, are commonly used in the treatment of hypertension, ischemic heart disease, and arrhythmia (reviewed in Sproat and Lopez1). However, $\beta$-blockers cause several side effects, which are usually associated with their central nervous system (CNS) reaction (eg, sleep disturbance, depression, impotence, dizziness, and fatigue) and $\beta_2$-adrenergic antagonistic activity (eg, increase in peripheral vascular resistance, worsening of asthma symptoms). In addition, because of their short half-life (3 to 10 hours), $\beta$-blockers must be taken daily to be effective. Because a cardiovascular disease such as hypertension is a life-long disorder, longer-lasting treatment without side effects would be desirable.

Although the precise mechanism underlying the antihypertensive effects of $\beta$-blockers remains unclear, it is generally accepted that they antagonize the $\beta_1$-AR activity in heart and kidney, decreasing cardiac output and plasma renin activity.1 We have designed a new approach to $\beta_1$-blockade that reduces the number of receptors. Antisense oligonucleotides (AS-ODN) or antisense DNA designed specifically against $\beta_1$-ARs might represent a new class of $\beta$-blockers. Antisense technique, through a number of mechanisms,2 can effectively downregulate the expression of target proteins. Clinical trials using antisense in targeting AIDS,3 cancer,4 and other genetic and acquired diseases5 indicate their potential clinical usefulness. The antisense approach has several potential advantages over $\beta$-blockers. First, the specificity of AS-ODNs is based on DNA sequence. Second, AS-ODNs do not have direct CNS effects, because of the negligible transport of these highly polar molecules through the blood-brain barrier.6 Third, antisense elements tested in different systems produce long-term effects after single treatment.7 This prolonged effect can be attributed to 2 features of AS-ODN. One is the extended half-life of chemically modified ODN. The half-life...
of 15- to 20-mer phosphorothioated ODN is 20 to 50 hours in rats and mice after intravenous injection.8,9 The other is associated with the nature of antisense inhibition, which provides a delayed yet prolonged blockade of target proteins distinct from the direct competitive antagonists currently available.

We hypothesize that β1-antisense, through specific inhibition of β1-AR expression, will decrease the functional sensitivity of β1-AR-mediated responses in the face of sympathetic activation and thereby achieve an antihypertensive effect. In this study, we designed an AS-ODN complementary to rat β1-AR mRNA and tested its ability to inhibit β1-AR density and function in the heart and to reduce BP in spontaneously hypertensive rats.

Methods

Antisense Design and Administration

Antisense ODN and inverted ODN control were 15-mer and targeted to the AUG start codon of rat β1-AR mRNA.10 The sequence of AS-ODN is 5′-CCCGGCCCCATCCAAGCA-3′, and the inverted ODN is 5′-AGCCGTACCCGCGCC-3′. This AS-ODN was chosen from 6 AS candidates targeted to different regions of β1-AR mRNA on the basis of the intensity of cardiac β1-AR inhibition and reduction of BP in SHRs. These oligonucleotides were modified by backbone phosphorothioation. ODNs delivered with cationic liposomes were injected into the tongue vein.

Preparation of Liposomes and ODN/Liposome Complex

The cationic lipid 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) was mixed with helper lipid 1,2-dioleoylphosphatidylethanolamine (DOPE, Avanti Polar Lipids) at a 1:1 molar ratio, briefly sonicated, and stored at 4°C until use. The average diameter of liposomes is 200 to 350 nm.11 ODN/liposome complex was prepared on the day of use by mixing the desired amounts of ODNs with DOTAP/DOPE to the final DNA concentration of 300 μmol/L. This AS-ODN was chosen from 6 AS candidates targeted to different regions of β1-AR mRNA on the basis of the intensity of cardiac β1-AR inhibition and reduction of BP in SHRs. Each ODN was 20 to 50 hours in vivo.

Membrane Preparation and β-AR Binding Assay

Four days after intravenous injection of saline (n=6) or 1 mg/kg inverted ODN (n=6) or 2, 4, 10, or 18 days after injection of 1 mg/kg β1-AS-ODN (n=24), animals were euthanized, and membranes were prepared from heart ventricles as previously described.12 For saturation experiments, 100 μg membrane protein was incubated in triplicate with 6 concentrations of [3H]-isoproterenol and 150 μmol/L CGP20712A (RBI), respectively. Then the reaction mixture was passed through Whatman GF/B glass fiber filter using a Brandel harvester, and the bound radioactivity was counted for 1 minute.

Tissue Preparation and Quantitative Autoradiography

Four days after injection of 1 mg/kg β1-AS-ODN (n=6) or saline (n=6), rats were killed, and tissues were removed and frozen in dry ice. Coronal sections of brain, horizontal sections of heart, and sagittal sections of kidney (20 μm) were cut on a cryostat (Microm) at −20°C and mounted on microscope slides. Every seventh slide was stained with hematoxylin and eosin for histology. Tissue sections were preincubated in Krebs buffer (mmol/L: NaCl 118.4, KCl 4.7, MgSO4 1.2, CaCl2 1.27, and NaH2PO4 10.0, pH 7.1) containing 0.1 mmol/L GTP, 0.1 mmol/L ascorbic acid, and 10 μmol/L PMSF for 30 minutes at 25°C. Sections were then incubated in Krebs buffer containing 0.1 mmol/L ascorbic acid and 10 μmol/L PMSF with 100 pmol/L ICYP at 25°C for 150 minutes in the presence of 1 μmol/L, L- (−)-propranolol, 100 nmol/L ICI 118,551 (β1-selective antagonist), or 100 nmol/L CGP20712A (β2-selective antagonist) to distinguish nonspecific, β1-, and β2-binders. Labeled sections were rinsed in the same buffer, followed by two 15-minute washes at 37°C in buffer and rinsed in distilled water at 25°C.13 Dried sections were then exposed to x-ray films. The images were quantified with a computerized image analysis system (MCID, Imaging Research) and normalized with125I standards. Nonspecific binding was <10% of total binding.

Determination of Effects of β1-AS and Atenolol on Cardiovascular Parameters in Response to β-Stimulation

Langendorff Heart Perfusion

Forty-eight hours after injection of 1 mg/kg β1-AS-ODN (n=9) or inverted ODN (n=6), SHRs were anesthetized and killed. Hearts were quickly removed and perfused via the aorta with oxygenated Krebs buffer (118 mmol/L NaCl, 18.75 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.25 mmol/L CaCl2, 11.1 mmol/L glucose, and 0.01 mmol/L EDTA) at a constant flow of 7.0 mL/min at 36°C. Coronary perfusion pressure was measured via a catheter placed proximal to the aorta and connected to a pressure transducer (Gould Statham P23ID). A latex balloon filled with water and connected to the pressure transducer was inserted into the left ventricle through the left atrium to measure left ventricular end-diastolic pressure (LVDP), left ventricular systolic pressure (LVSP), and developed left ventricular pressure (dLVP) (dLVP=LVSP−LVDP). LVDP and LVSP were recorded continuously on a 4-channel recorder (Astro-Med). After baseline values for dLVP and heart rate (HR) were stable for 5 minutes, isoproterenol (ISO, nonspecific β-agonist) was given at 0.01, 0.025, 0.05, and 0.12 μmol/L at 10-minute intervals so as to avoid the effect of tachyphylaxis.

Telemetric Monitoring of Live Animals

The effects of β1-AS-ODN and atenolol on cardiac dp/dtmax, HR, and systolic blood pressure (SBP) were compared in the same group of SHRs (n=4). Two days after catheterization of the jugular vein, control values were taken and 1 mg/kg β1-AS-ODN was injected.
Forty-eight hours later, rats were tested for the effect of β₁-AS-ODN. The rats were allowed to recover until all the cardiovascular parameters returned to control values. Then 1 mg/kg atenolol (β₁-selective antagonist) was injected, and rats were tested 30 minutes later. For β₁-stimulation, SHRs were infused with dobutamine (β₁-selective agonist) through a jugular vein catheter at 5, 10, 20, and 40 μg/g/min. Each dose was given for 5 minutes continuously and at 1-hour intervals until all the cardiovascular parameters returned to baseline values so as to avoid the effect of tachyphylaxis. BP and HR were sampled every 1 minute. dP/dt max was calculated from the slope of the rising pressure curve and determined every 1 minute. The difference between values at each dose and baseline was denoted as Δ.

BP Monitoring

Telemetry
Each rat cage was placed on a receiver (RLA1020, Data Sciences) for measurement of cardiovascular parameters. Data were collected with a computer-based data acquisition program (Dataquest LabPRO3.0; Data Sciences). BP and HR were measured every 10 minutes and averaged every 1 to 24 hours. Before treatment, SHRs were monitored for a week to get a stable baseline.

Tail Cuff
Rats were warmed for 20 to 30 minutes in cages on heating pads. The temperature was controlled at 35°C to 37°C. Then the rats were placed in a plastic restrainer kept at 37°C. A pneumatic pulse sensor was attached to the tail. After cuff inflation, SBP was determined as the first pulsatile oscillation on the descending side of the pressure curve. HR was determined by manual counting of pulse numbers per unit time. BP and HR were recorded by a Narco physiograph. Data values of each rat were taken as an average of at least 4 stable readings. Baseline was determined by averaging 3 days of measurements before antisense administration.

Statistical Analysis
Values were expressed as mean±SEM. The difference was considered statistically significant at P<0.05. An unpaired t test was used to compare Bmax, dLVP, and BP in 2 groups. One-way repeated-measures ANOVA and Tukey test were used to compare Bmax, dLVP, and BP in 2 groups. Pearson product-moment correlation was used to assess the relationship between β₁-AR Bmax and dLVP.

Results

Effect on Cardiac β-AR Density
Figure 1A shows that a single intravenous injection of 1 mg/kg β₁-AS-ODN delivered with cationic liposomes at a 1:2.5 molar ratio significantly reduced the β₁-AR density in the SHR hearts for 18 days (P<0.02). The drop was at its maximum of 47% on day 4, 33% on day 10, and maintained at 29% on day 18. In contrast, there was no significant change in the Bmax of β₂-ARs. The Kd of both subtypes remained unaltered (Table). Consequently, the β₁/β₂ subtype ratio in the ventricles was diminished from ~70/30 to ~50/50 by β₁-AS-ODN. Inverted ODN had no effect on either subtype (Figure 1B).

Effect on Cardiac Contractility and HR in Response to β-Stimulation
Forty-eight hours after injection of 1 mg/kg β₁-AS-ODN, the cardiac inotropic and chronotropic responses to β-stimulation were determined in SHRs in vitro and in vivo.

First, isolated hearts were perfused with incrementing doses of ISO, which enhanced HR and contractility via activating β-ARs. The dLVP-ISO dose-response curve, which reflected the positive inotropic effect of ISO, was significantly shifted downward by β₁-AS-ODN (P<0.02). HR was not significantly decreased, except at 1 point, ie, 0.01 μmol/L ISO (P<0.05) (Figure 2).

An in vivo test was performed in conscious SHRs monitored by radiotelemetry. β₁-AS-ODN significantly (P<0.02) dampened the increase in dP/dt max in the face of dobutamine (β₁-selective agonist) (Figure 3A). The change in HR was not significantly reduced (Figure 3B), which echoed the results in isolated perfused hearts. Conversely, the response of BP to dobutamine was biphasic (Figure 3C). Dobutamine elevated SBP by 5 to 8 mm Hg at low infusion speed and reduced SBP at higher speed, probably as a result of the partial β₂-agonistic activity of dobutamine at high doses and the consequent vasodilatory effect on BP. Figure 3 also compares the results with β₁-AS-ODN and atenolol (β₁-selective antagonist). Relative to β₁-AS-ODN, 1 mg/kg atenolol produced a more profound decline in dP/dt max and ΔHR during a 5-hour period of time after injection. Moreover, it reduced basal dP/dt max (2450±295 mm Hg/s versus control, 2937±277 mm Hg/s) and caused bradycardia (295±12 bpm versus control, 365±8 bpm).
Table 1. Characteristics of SHRs 4 Days After Treatment With Saline, Inverted ODN, or β₁-AS-ODN

<table>
<thead>
<tr>
<th>Group</th>
<th>Total (β₁ + β₂)</th>
<th>β₁</th>
<th>β₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₒ, pmol/L</td>
<td>Bmax, fmol/mg</td>
<td>Kₒ, pmol/L</td>
</tr>
<tr>
<td>Saline</td>
<td>55.4±6.7</td>
<td>27.6±0.9</td>
<td>64.7±5.4</td>
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<tr>
<td>Inverted ODN</td>
<td>57.1±3.5</td>
<td>26.3±3.0</td>
<td>66.0±1.0</td>
</tr>
<tr>
<td>β₁-AS-ODN</td>
<td>37.0±1.0*</td>
<td>19.8±1.8*</td>
<td>60.5±3.4</td>
</tr>
</tbody>
</table>

Data represent mean±SEM of each group (n=6 to 10). *P<0.01 vs saline control.

Effect on BP of SHRs

Figure 4 shows the effects of a single injection of 1 mg/kg β₁-AS-ODN on the BP of SHRs measured by the tail-cuff method. AS-ODN was delivered with cationic liposomes at different molar ratios of DNA/lipid, ie, 1:0.5 and 1:2.5. β₁-AS-ODN delivered with liposomes at a 1:0.5 ratio diminished SBP for 8 days. The maximum drop was 38±5 mm Hg. When the molar ratio was increased to 1:2.5, this hypotensive effect was drastically prolonged to 20 days. No effect was seen with inverted ODN.

To compare the effects of β₁-AS-ODN and atenolol, radiotelemetry was used to monitor BP and HR on a regular basis. β₁-AS-ODN 1 mg/kg delivered with liposomes at a 1:0.5 ratio produced a maximum drop of 15 mm Hg in mean BP (Figure 5A). The antihypertensive effect lasted for 8 days, which was consistent with the results measured with the tail cuff. HR was not significantly altered (Figure 5B). In contrast to AS-ODN, although the onset of the hypotensive effect caused by 1 mg/kg atenolol occurred as early as 20 minutes after injection, it lasted for only 10 hours (Figure 6A). In addition, atenolol caused considerable bradycardia up to an average of −75 bpm (Figure 6B).

Effect on β-AR Distribution in Brain, Heart, and Kidney

Quantitative autoradiography of 6 to 18 tissue slices in brain, heart, and kidney was analyzed 4 days after intravenous β₁-AS-ODN administration (Figure 7). No changes in the distribution of β-ARs in the forebrain and brain-stem regions were detected. This indicated the absence of antisense effect on the β-AR expression in CNS. However, β₁-AS-ODN significantly (P<0.05) reduced β₁-AR density in cardiac ventricles (from 30.2±2.1 to 20.6±2.5 fmol/mg) and renal cortex (from 26.4±3.1 to 17.4±3.3 fmol/mg). This was consistent with the binding results. β₂-ARs were not affected in any tissues (data not shown).

Discussion

This study was designed to compare the effects of a novel β₁-AS-ODN on high BP in a model of hypertension with a currently used β-blocker. β₁-AS-ODN knocked down β₁-adrenergic activity, resulting in long-term attenuation of BP. The results indicated that β₁-AS-ODN reduced β₁-AR density and cardiac contractility after β₁-stimulation in vitro and in vivo and lowered high BP of SHRs. These findings are consistent with our hypothesis that β₁-AS, through the inhibition of β₁-AR expression, is able to render heart, kidney, and other tissues less sensitive to sympathetic activation, which is a major contributing factor in high BP. A single injection of β₁-AS-ODN effectively decreased the cardiac β₁-AR density, which was accompanied by diminished ven-
tricular contractility and cardiac output in response to $\beta$-stimulation. The antihypertensive effect in SHRs was up to a 38 mm Hg reduction lasting as long as 20 days.

Treatment of $\beta_1$-AS-ODN reduced cardiac $\beta_1$-AR density by $\approx 50\%$ in 4 days. Considerable variation is reported in the literature on the half-life of $\beta$-ARs. 12,14,15 From our results with the AS-ODN inhibition, we deduce that the half-life of cardiac $\beta_1$-ARs is $\approx 2$ to 4 days to allow for 50% reduction of $\beta$-AR density within 4 days.

We noted that both tail-cuff and telemetry measures of BP showed a significant drop after antisense treatment. SHRs responded to $\beta_1$-AS-ODN to a greater degree of hypotension when subjected to tail cuff versus telemetry. In addition, the baseline measured with the tail cuff was consistently higher than that with telemetry by 20 to 30 mm Hg in the same rats. Bazil et al16 reported a similar phenomenon. They compared the cardiovascular parameters recorded by telemetry, tail cuff, and arterial catheter and observed a more sensitive hypotensive effect of captopril with tail cuff. Tail-cuff measurement of BP involves warming and restraint of rats. It is conceivable that $\beta_1$-AS, through the suppression of sympathetic activity, can decrease the BP of animals under stress more effectively.

Cationic liposomes are effective vehicles for gene delivery. It has been shown that liposome entrapment not only improves the cellular uptake of DNA but also protects DNA from degradation and extends its circulation time.17,18 Numerous factors influence the efficiency of cationic liposome-mediated intravenous gene delivery, such as DNA/lipid ratio, selection of lipids, and preparation procedure.18,19 We chose a widely used lipid formula, DOTAP/DOPE, to deliver $\beta_1$-AS-ODN at 4 molar ratios (data for ratios 1:1.5 and 1:3.5 are not shown). The optimal ratio of 1:2.5 was determined on the basis of the duration and magnitude of the antihypertensive effects. A profound and prolonged fall in BP of 38 mm Hg up to 20 days was achieved at this ratio, which followed the reduction in $\beta_1$-AR binding to a maximum of 47% at day 4, 33% at day 10, and 29% at day 18. This implies that $\beta_1$-AS-ODN effectively inhibits the functionally active receptors involved in the BP.

The blood-brain barrier formed by capillary endothelia is permeable only to small lipophilic molecules with a molecular weight of <600 Da (reviewed by Partridge20). Owing to their high hydrophilicity, ODNs undergo negligible transport through blood-brain barrier and have very limited access to CNS.6 The cellular and organ distributions of DNA/liposome
complexes with fluorescent labeling were previously studied in mice after intravenous injection, and the results indicated that the complexes were taken up primarily by capillary endothelial cells in most of the peripheral organs, including lung, heart, kidney, and spleen, but were absent in the brain. In our study, autoradiography in brain revealed no detectable changes in the expression and distribution of $\beta_1$-ARs after intravenous $\beta_1$-AS-ODN injection. This provided further evidence that our antisense approach did not have CNS effects.

High specificity based on gene sequence has made antisense an increasingly useful tool in numerous studies and clinical trials. Its success is manifested by the recent approval of the first antisense drug, Vitravene, by the Food and Drug Administration. However, sequence-independent interactions have also been reported with AS-ODNs. High doses are usually responsible for the nonspecific effects, but another possible reason is that currently available databases do not cover every gene; thus, homology comparison by BLAST search may not guarantee sequence specificity of AS-ODNs. In our study, $\beta_1$-AS-ODN inhibited $\beta_1$-AR expression without changing $\beta_2$-ARs. Although we hypothesize that this is due to the specificity of the $\beta_1$-AS-ODN sequence, we recognize that other possibilities remain, such as indirect effects on regulatory mechanisms.

In patients with chronic heart failure, the severity of the disease closely relates to the decrease in cardiac $\beta$-AR density and functional responsiveness. In SHRs treated with $\beta_1$-AS-ODN, we observed a marked attenuation of the $\beta_1$-AR–mediated positive inotropic response in vitro and in vivo, concurrent with the diminished cardiac $\beta_1$-AR level. Therefore, our results indicated a positive correlation between cardiac $\beta_1$-AR number and functional sensitivity (correlation coefficient $>0.90$, $P<0.01$).

Despite the large decrease in BP, no reflex tachycardia was observed after antisense treatment. However, the suppressive effect of $\beta_1$-AS-ODN on HR was less significant than its negative inotropic response. The reason for this is still under investigation. Several possibilities can be considered. First, $\beta_1$-AS-ODN did not affect $\beta_2$-ARs, which played an important role in the regulation of HR, although it was not involved in the cardiac contraction. Second, antisense inhibition of $\beta_1$-AR expression is gradual and less extensive than with $\beta$-blockers. It is also possible that $\beta_1$-ARs may have a larger reserve for controlling HR than contractility. Finally, preliminary evidence in our laboratory suggests that cardio-
myocytes preferentially take up AS-ODN, whereas pacemaker cells are less efficient (unpublished data).

The cardiovascular effects of \( \beta_1 \)-AS-ODN were compared with those of a hydrophilic \( \beta_1 \)-selective antagonist, atenolol. \( \beta_1 \)-AS-ODN showed advantages over atenolol in reducing BP and maintaining normal HR. Although the onset of \( \beta_1 \)-AS-ODN action was slower than atenolol, it lasted much longer, 20 days compared with <1 day with atenolol. Furthermore, \( \beta_1 \)-AS-ODN did not affect HR, whereas atenolol caused appreciable bradycardia. Bradycardia is a common complaint by patients taking this drug. Atenolol also reduced BP caused appreciable bradycardia. Bradycardia is a common complaint by patients taking this drug. 

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