Reduction of Cold-Induced Hypertension by Antisense Oligodeoxynucleotides to Angiotensinogen mRNA and AT₁-R Receptor mRNA in Brain and Blood

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Abstract—Rats exposed chronically to mild cold (5°C/41°F) develop hypertension and cardiac hypertrophy. This provides a unique model of hypertension that is environmentally induced. The blood renin-angiotensin system (RAS) has been shown to play a role in both initiating and maintaining the high blood pressure (BP) in cold-induced hypertension. The mechanism also appears to involve both the tissue and brain RAS because there is increased mRNA for angiotensinogen (AGT) and angiotensin type 1 (AT₁) receptors in brain and peripheral tissues, an increased spontaneous drinking response, and an increased dipsogenic response to acute administration of angiotensin II (Ang II) in cold-treated rats. Antisense oligodeoxynucleotides (AS-ODN), targeted to the RAS, have been shown to reduce BP in spontaneously hypertensive rats. Therefore, we injected AS-ODN in rats with cold-induced hypertension to test whether antisense inhibition was effective in reducing this nongenetic nonsurgical hypertension. Sprague-Dawley rats were made hypertensive by cold exposure and injected intracerebroventricularly with AS-ODN to AGT mRNA (n=6) or AT₁ receptor mRNA (n=6). Systolic BP was recorded by tail cuff 24 hours later for 2 or 7 days, respectively. Systolic BP decreased significantly in response to AGT–AS-ODN (40±6 mm Hg, P<0.01) within 1 day after injection and to AT₁ receptor–AS-ODN (P<0.05) for 3 days after injection. The maximum decrease was 41±10 mm Hg. Systolic BP then gradually increased to the preinjection level. The spontaneous drinking response to cold treatment also decreased significantly (P<0.05) after AGT–AS-ODN or AT₁ receptor–AS-ODN intracerebroventricular injection. Intracardiac injection of AT₁–AS-ODN (n=6) reduced systolic BP by 36±8 mm Hg (P<0.05) and decreased AT₁ receptor mRNA (as measured by autoradiography in aorta, adrenal glands, and kidneys 24 hours after injection). These data show that AS-ODN reduces BP in cold-induced hypertension and that the hypertension involves both peripheral tissues and central RAS in addition to blood-borne RAS mechanisms. (Hypertension. 1998;31:1317-1323.)

Key Words: hypertension, cold-induced ■ angiotensinogen ■ angiotensin II ■ RNA ■ receptors ■ antisense elements

Rats exposed to mild cold (5°C/41°F) develop hypertension.1–4 This CIH is a nongenetic, nonsurgical model for studying hypertension in rats. Our previous studies have shown that the blood-borne RAS either prevents or attenuates CIH.5,6 The mechanism also appears to involve both peripheral tissue and central RAS because there is increased mRNA for AGT and/or AT₁-R in brain and peripheral tissues,7 increased spontaneous drinking response, and increased dipsogenic response to acute administration of Ang II in cold-treated rats. Furthermore, studies also suggest that there is a direct correlation between the state of receptors for Ang II in the diencephalon and development of hypertension, ie, upregulation of receptors for Ang II has been linked to the induction of both deoxycorticosterone acetate–salt and spontaneously induced hypertension.5–13 Tests of the dipsogenic responsiveness to Ang II, spontaneous drinking response to cold, and AT₁-R mRNA in brain show much greater responses in cold-treated rats.6 This suggests that the receptors for Ang II are upregulated in rats exposed chronically to cold. Additional studies have shown an increased amount of mRNA for AGT, the substrate for the RAS, in the brains of cold-treated rats compared with rats kept at room temperature as controls. This raises the possibility that both increased production of Ang II and upregulation of Ang II receptors contribute to the elevation of BP during exposure to cold. An increased amount of mRNA of AGT and/or AT₁-R in peripheral tissues (liver, adrenal glands, and aorta) of cold-treated rats suggests that an overactive peripheral tissue RAS is involved in CIH as well. AS-ODN, targeted to AGT mRNA or AT₁-R mRNA, have been shown to reduce BP in SHR, which demonstrates that AS-ODN are specific and reduce an overactive RAS.14–18 Therefore, to assess more directly the role of the central RAS in CIH, AS-ODN to AGT mRNA and to AT₁-R mRNA were
administered centrally, and their effects on BP and spontaneous drinking in chronically cold-exposed rats were determined. To assess the role of the peripheral RAS in CIIH, AS-ODN to AT\(_1\)-R mRNA was administered by intracardiac injection, and its effect on BP and AT\(_1\)-R in chronically cold-exposed rats was determined. We hypothesized that central injection of AS-ODN to AGT or AT\(_1\)-R mRNA would decrease BP and spontaneous drinking response, and that a peripheral injection of AS-ODN to AT\(_2\)-R mRNA would decrease BP and AT\(_1\)-R binding in cold-treated rats.

**Methods**

**Animal Model**

Forty-two adult male Sprague-Dawley rats (225 to 250 g) were acquired from Harlan. The rats were kept in individual cages in a laboratory in which rats exposed to chronic cold developed hypertension.\(^1\)–\(^4\) The rats were deeply anesthetized with sodium pentobarbital (65 mg/kg body wt IP) and perfused transcardially with 0.9% saline. The tissues were removed and kept frozen at \(-20^\circ\text{C}\) until cryostat sectioning into 20-\(\mu\)m sections. The sections were mounted onto gelatin-coated slides. The slides were preincubated in assay buffer (150 mmol/L NaCl, 50 mmol/L sodium phosphate, 1 mmol/L EDTA, 0.1 mmol/L bacitracin, pH 7.2) for 30 minutes and then incubated with the same buffer with the addition of 500 pmol/L \[^{125}\text{I}\]-\text{Sar}^1,\text{Ile}^8-\text{Ang II} for 2 hours. The sections were incubated with the radioligand in the presence or absence of 1 mmol/L Ang II to determine nonspecific and total receptor binding. AT\(_1\)-R or AT\(_2\)-R binding was determined in the presence of 1 mmol/L of either PD123319 or losartan. The sections were washed and dried. Autoradiograms were generated by exposing the slides to x-ray films for 1 to 4 weeks. They were analyzed by autoradiographic densitometry (Image Systems Inc). The photos were taken directly from the image analysis system. Optical densities of the autoradiograms were determined by computerized microdensitometry, and the results are expressed in femtomoles per gram of protein after comparison with the \(^{125}\text{I}\)-standard.\(^21\)

**Statistical Analysis**

The data for SBP were analyzed by repeated measures one-way ANOVA using the Newman-Keuls procedure to assess the significance of differences between two means. For ICV injection, the data for SBP and water intakes were analyzed by a repeated measures two-way ANOVA using the Newman-Keuls procedure to assess the significance of differences between two means. For intracardiac injection, SBP and AT\(_1\)-R and AT\(_2\)-R binding were compared by Student’s \(t\) test. A value of \(P<0.05\) was considered significant.

**Cold-Induced Hypertension**

SBP increased during 5 weeks of exposure to cold (Figure 1). At 1, 2, 3, 4, and 5 weeks of cold exposure, SBP was 121 ± 1, 146 ± 3, 149 ± 3, 157 ± 3, and 165 ± 3 mm Hg, respectively. By the second week of cold exposure, SBP was significantly greater than that before cold exposure (\(P<0.01\)). The difference was pronounced during the fifth week of cold exposure. This result is consistent with previous studies from this laboratory in which rats exposed to chronic cold developed hypertension.\(^1\)–\(^4\)
Effect of Central Injection of AS-ODN to AGT mRNA on SBP and Spontaneous Drinking Response

Blood Pressure
A highly significant decrease in SBP was observed in the AS-ODN–treated rats. The result of injecting AS-ODN to AGT mRNA was that SBP was reduced from a hypertensive level (157±6 mm Hg) to a normotensive level (117±3 mm Hg) at 24 hours after injection in the AS-ODN–treated rats. Figure 2 shows the effect of central injection of AS-ODN on SBP in cold-treated rats. The SBP of AS-ODN–treated rats was significantly decreased to 117±6 mm Hg at 24 hours after injection (P<0.01) and to 139±5 mm Hg at 48 hours after injection (P<0.05). These pressures were significantly lower than those of the SCR-ODN– or artificial CSF–treated groups. There was no significant difference in the response to SCR-ODN injection (from 152±6 to 155±6 mm Hg at 24 hours after injection, to 150±8 mm Hg at 48 hours after injection, P>0.05) or to artificial CSF injection (from 47±10 to 50±8 mL/d at 24 hours after injection, to 61±8 mL/d at 48 hours after injection, P>0.05). The AS-ODN significantly reduced spontaneous drinking compared with SCR-ODN or artificial CSF administration at the same time intervals (P<0.05).

Effect of Central Injection of AS-ODN to AT₁-R mRNA on SBP and Drinking Response

The result of injecting AS-ODN to AT₁-R mRNA was that SBP and water intake were reduced in the AS-ODN–treated rats.

Drinking Response
The spontaneous drinking response was also significantly decreased by AS-ODN. Chronic cold exposure increases spontaneous drinking response (the percent increase from control is 10% at 1 week, 19% at 3 weeks, and 41% at 5 weeks during cold exposure), probably due to the increased activity of brain RAS.8,22 Figure 3 shows the effect of central injection of AS-ODN on spontaneous drinking response in cold-treated rats. The drinking response of AS-ODN–treated rats was significantly decreased from 49±3 to 27±4 mL/d at 24 hours after injection (P<0.01) and to 30±6 mL/d at 48 hours after injection (P<0.01). There was no significant difference in the response to SCR-ODN injection (from 59±14 to 65±6 mL/d at 24 hours after injection, to 68±6 mL/d at 48 hours after injection, P>0.05) or to artificial CSF injection (from 47±10 to 50±8 mL/d at 24 hours after injection, to 61±8 mL/d at 48 hours after injection, P>0.05). The AS-ODN significantly reduced spontaneous drinking compared with SCR-ODN or artificial CSF administration at the same time intervals (P<0.05).
(from 172±6 to 138±5 mm Hg, P<0.05) after the single dose of 50 μg (25 μg/μL). The SBP remained decreased by >30 mm Hg, and the maximum effect was seen 72 hours after the injection (to 131±5 mm Hg, P<0.01). The SBP gradually increased and reached the control level by day 5 to 6 after injection. The controls, artificial CSF and SCR-ODN, produced no significant reduction in SBP.

Drinking Response

Figure 5 shows the effect of central injection of AS-ODN on water intake in cold-treated rats. Cold increases spontaneous water intake. The drinking after AS-ODN administration was significantly decreased from 58±4 to 41±5 mL/d at 24 hours after injection (P<0.05). This inhibitory effect on drinking behavior was also present at 48 hours after injection (41±4 mL/d, P<0.05). There was no difference in water intake after SCR-ODN (from 60±4 to 51±4 mL/d at 24 hours after injection, to 47±4 mL/d at 48 hours after injection, P>0.05) or artificial CSF (from 58±6 to 50±6 mL/d at 24 hours after injection, to 61±6 mL/d at 48 hours after injection, P>0.05). The AS-ODN significantly reduced drinking compared with SCR-ODN or artificial CSF administration at the same time intervals (P<0.05).

Effect of Peripheral Injection of AS-ODN to AT1-R mRNA on SBP and AT1-R Binding

To test the effectiveness of antisense inhibition delivered systemically, injections of AS-ODN to AT1-R mRNA were given. The effect of this AS-ODN inhibition was a significant reduction in SBP. A single injection with the receptor AS-ODN (100 μg in 100 μL saline) resulted in a significant fall in SBP of 35±5 mm Hg (P<0.01) in cold-treated rats (Figure 6) 24 hours after injection compared with SCR-ODN administration at the same time (**P<0.01; n=6). To examine the tissues for changes in AT1-R binding at 24 hours after injection, no further time points were measured.

Figure 6. Effect of peripheral injection of AT1-R mRNA AS-ODN on SBP in cold-treated rats. Rats were administered 100 μg AS-ODN or SCR-ODN intracardially. SBP of the antisense-treated rats was significantly decreased to a normotensive level 24 hours after injection compared with SCR-ODN administration at the same time (**P<0.01) (n=6).

Figure 7. 125I-Sar1,Ile8-Ang II autoradiography analysis of multiple transverse sections of aorta, adrenal glands, and kidneys of cold-treated rats that received intracardiac injection in Figure 6. Columns indicate AT1-R binding; error bars represent standard error. *P<0.05.
Autoradiography of Peripheral Tissues

 Autoradiography showed that treatment with AS-ODN resulted in a significant decrease in AT₁-R binding in aorta, adrenal glands, and kidneys when compared with the SCR-ODN treatment. These results are summarized in Figure 7 and Figure 8A through 8F. The decreases observed with 100 μg AS-ODN injection were 92% in aorta, 42% in adrenal glands, and 28% in kidneys. No decrease in AT₂-R binding was observed (data not shown).

Figure 8. Specific binding of AT₁-R in various tissues. After AT₁-R mRNA AS-ODN administration, AT₁-R binding was decreased compared with after SCR-ODN administration (color code shows calibration for femtomoles per gram). A, Aorta section of rats given SCR-ODN; B, aorta section of rats given AS-ODN to AT₁-R mRNA; C, adrenal gland section of rats given SCR-ODN; D, adrenal gland section of rats given AS-ODN to AT₁-R mRNA; E, kidney section of rats given SCR-ODN; and F, kidney section of rats given AS-ODN to AT₁-R mRNA.
Antisense Inhibition of Cold-Induced Hypertension

Discussion

Rats exposed to cold for several weeks develop a significant BP elevation. This takes 3 to 4 weeks of chronic exposure to cold. The mechanism(s) for induction of hypertension after chronic exposure to cold is incompletely understood and is presently under study. However, recent studies have implicated both the central RAS and peripheral RAS as contributors. The peripheral RAS includes both the classic blood-borne RAS and the tissue RAS. There is an increase in mRNA for AGT and AT₁-R in brain and peripheral tissues and increased spontaneous drinking response in cold-exposed rats that respond to Ang II administration with an increased dipsogenic response. The results of the present study support a role for the central and peripheral tissue RAS in the maintenance of CIH in that these experiments illustrate the viability of antisense inhibition in vivo for reduction of BP in a nongenetic, environmentally induced model of hypertension. Both antisense to AT₁-R and AGT mRNA were successful in reducing BP centrally and antisense to AT₁-R mRNA in reducing BP peripherally. Reduction in BP was correlated to an inhibition of spontaneous drinking response and to a reduction of AT₁-R binding in peripheral tissues, respectively.

The results provide evidence that the brain RAS is involved in CIH. The AS-ODN to AT₁-R mRNA inhibited the physiological effects, pressor and drinking responses, produced by central administration of Ang II. The results of this study are in keeping with the hypothesis that inhibition of mRNA translation for Ang II or AT₁-R in the brain can reduce BP of rats with CIH. When coupled with our previous results showing that mRNA for the Ang II, AT₁-R, spontaneous-drinking, and dipsogenic responses to administration of Ang II is increased in cold-treated rats, a role for the central RAS in this type of experimentally induced hypertension is indicated.

The data presented here confirm the effectiveness of antisense inhibition in reducing hypertension. In previous studies, we have used the same AS-ODN designs to lower BP in a genetic model of hypertension, the SHR. Gyurko et al. showed that central injection of AS-ODN decreased BP in SHR by blocking the protein synthesis of central AT₁-R. Wielbo et al. showed that AS-ODN targeted to AGT mRNA reduced hypertension in SHR when given centrally or systemically. Morishita et al. also found similar AS-ODN to AGT mRNA to be effective in transient reduction of BP in SHR. The prolonged reduction in high BP in the present experiment confirms the pattern of effect of AS-ODN to AT₁-R IVC observed in SHR. The present results expand on these studies by demonstrating the effectiveness of antisense inhibition in a different model of hypertension. The CIH model is nonsurgical and nongenetic; it is an environmentally induced model of hypertension. Thus, antisense administration lowers BP in genetic- and cold-induced hypertension. The results also confirm the findings of Meng et al. and Sakai et al. that the drinking response to Ang II (ICV) can be inhibited by AS-ODN to AT₁-R mRNA. The data suggest that the mechanism of hypertension development on exposure to cold is via increased production of AGT in the brain and consequently Ang II. Furthermore, the mechanism of CIH involves upregulated AT₁-R. Increased Ang II synthesis and increased AT₁-R induce the elevation of BP in cold-exposed rats and maintains it, once elevated.

Injections of AS-ODN into the heart produced a significant decrease in BP compared with injections of SCR-ODN. We used the intracardiac route for rapid dispersal of antisense in arterial blood. The results indicate that peripheral AT₁-R contributes to the hypertension in parallel with changes in brain RAS. Control injections of SCR-ODN had no effect in central or peripheral injections. Thus, the results demonstrated that systemic administration of AS-ODN is also a viable route and is effective for antihypertensive action.

To show that the mechanism of action responsible for these physiological effects is due to sequence-specific antisense inhibition, the amount of the targeted gene product, the AT₁-R, should be decreased. The results of the autoradiography experiments confirm a significant decrease in the number of AT₁-R in aorta, adrenal glands, and kidneys. Whether these are the sites of action for the effect on BP or other sites are involved cannot be resolved by this analysis. The results are not due to nonspecific effects. We have used every type of control in prior experiments, including scrambled, sense, vehicle, mismatch, inverted, and even antisense to ANP to produce the opposite effects of antisense to Ang II. In every case, the antisense to AT₁-R or to AGT mRNA produces a unique, specific response resulting in both physiological and molecular changes, indicating antisense inhibition. Earlier work by others using long oligos (22 mer or longer) with multiple repeats of G bases led to nonspecific binding. Sequences of 4 G cause self-annealing and misleading results. Our oligo design avoids these pitfalls by using shorter ODN (15 mer) and by excluding sequences with 4 or more G repeats.

In summary, we have shown that AS-ODN, targeted to the RAS, significantly reduced BP in a rat model of hypertension that is not genetically, surgically, or pharmacologically induced. The antisense administration centrally reduces BP for 3 to 4 days after a single injection in CIH rats, and peripheral administration was effective 24 hours after a single injection. These results demonstrate that both the central and peripheral RAS are involved in CIH.

Acknowledgments

This study was supported by National Institutes of Health MERIT award HL27334 and HL39154 (Dr Phillips). We wish to thank Howard Clark and Leping Shen for their expert technical assistance.

References


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Hypertension. 1998;31:1317-1323
doi: 10.1161/01.HYP.31.6.1317

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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