Abstract—The systemic renin-angiotensin system (RAS) plays an important role in blood pressure (BP) regulation during the development of 2-kidney, 1 clip (2K1C) hypertension. Its contributions decrease with time after constriction of the renal artery. During the chronic phase, the peripheral RAS returns to normal, but the hypertension is sustained for months. We hypothesized that in this phase the brain RAS contributes to the maintenance of high BP. To test the hypothesis, we studied the role of brain RAS by decreasing the synthesis of angiotensinogen (AGT) and the angiotensin II (Ang II) type 1a receptor (AT1R) with intracerebroventricular injections of antisense oligonucleotides (AS-ODNs). The response of systolic BP (SBP) to AS-ODNs to AGT mRNA was studied in 2K1C rats at 6 months after clipping, and the response to AS-ODNs to AT1R mRNA was studied at 10 months after clipping. Intracerebroventricular injection of AS-ODN-AGT (200 μg/kg, n=5) significantly decreased SBP (−22±6 mm Hg, P<0.05) compared with the sense ODN (n=5) and saline (n=3) groups. Intracerebroventricular injection of AS-ODN-AGT reduced the elevated hypothalamic Ang II level. The hypothalamic Ang II content in sense ODN and saline groups was significantly (P<0.05) higher than in the nonclipped group. Compared with inverted ODN, intracerebroventricular injection of AS-ODN-AT1R (250 μg/kg, n=6) significantly decreased SBP (−26±8 mm Hg, P<0.05) for 3 days after injection. This was a brain effect because intravenous AS-ODN-AT1R at a dose of 250 to 500 μg/kg did not affect SBP. These results suggest that the brain RAS plays an important role in maintaining the elevated SBP in chronic 2K1C hypertension. (Hypertension. 2001;37[part 2]:371-375.)

Key Words: rats ■ renin-angiotensin system ■ brain ■ hypertension, 2K1C ■ antisense

The renin-angiotensin system (RAS) plays an important role in blood pressure (BP) regulation during the development of renovascular hypertension. In the rat model of unilateral renovascular hypertension (the 2-kidney, 1 clip Goldblatt [2K1C] hypertensive rat), renal stenosis elevates the RAS. The contributions of RAS in this model vary depending on the time after constriction of the renal artery. In the acute phase of 2K1C hypertension, plasma renin activity rises, and the increase in BP is RAS dependent. Both plasma renin activity and plasma angiotensin II (Ang II) concentrations normalize in the chronic phase despite the continued high BP. To explain the antihypertensive effects of ACE inhibitors when the circulating RAS is not overly activated, the concept of tissue RAS has been used. It is proposed that in 2K1C hypertension, the RAS in various tissues is activated, and the products act in a paracrine fashion and are not detectable in plasma. An activation of tissue RAS in the acute phase of 2K1C hypertension has been extensively discussed. By contrast, few studies have examined the mechanism of maintained hypertension in the chronic phase, although clinically, this is much more relevant. Most renovascular hypertensive patients present with chronic high BP. We have shown that inhibiting the brain RAS in adult spontaneously hypertensive rats (SHR) significantly reduces hypertension. Baltatu et al have reported that the transgenic rat [TGR(ASrAoGEN)], which has permanent inhibition of brain-specific angiotensinogen (AGT) synthesis, shows a reduction of hypertension induced by a low-dose of Ang II. Therefore, we hypothesize that as the 2K1C hypertension progresses chronically, the brain RAS contributes to elevated BP in the absence of peripheral RAS elevation.

To test this hypothesis, we used antisense (AS) oligodeoxynucleotides (ODNs), which we previously designed to inhibit components of the brain RAS, including AGT mRNA and Ang II type 1a receptor (AT1R) mRNA. AS-ODN has some advantages over drugs: the gene-based design is more specific than a pharmacological antagonist, and the AS-ODN can be delivered in vivo and can produce prolonged effects rather than the transient effects of currently used drugs. These AS-ODNs produce a significant decrease in BP for 3 to 7 days with a single injection. Morishita et al
did not find an increase in the brain RAS at 16 weeks, but this may not have been long enough after clipping. In the present study, we investigate the role of brain RAS on BP regulation in chronic 2K1C rats at 6 and 10 months after clipping. To test the hypothesis, the rats received intracerebroventricular injections of AS-ODN to AT1 R or AT1 R mRNA. We also examined the activity of RAS by measuring Ang II levels in the brain tissue and plasma.

Methods

Animals and Surgery
Experiments were performed in adult male Sprague-Dawley rats (250 to 270 g, n = 24). All experimental procedures were approved by the Animal Care Committee of the University of Florida. Surgical procedures were performed under anesthesia induced by subcutaneous injection of a mixture of ketamine, xylazine, and acepromazine (50, 5, 1 mg/kg, respectively). The left renal artery was exposed, and a silver clip (0.2-mm internal diameter) was applied over the left renal artery. One week after recovery from surgery, systolic BP (SBP) was measured by the tail-cuff method. Throughout the study, rats were given tap water to drink and standard rat chow to eat ad libitum and were kept on a 12-hour light and dark cycle. Two groups of chronic 2K1C rats were tested: the first group at 6 months and the second group at 10 months after clipping of the renal artery. One week before testing, the rats were again anesthetized and placed in a stereotaxic frame. A guide cannula (23 gauge) was implanted into the lateral cerebral ventricle by stereotaxic coordinates (1.5 mm posterior to the bregma, 1.7 mm lateral to the midline, and 5.0 mm below the skull surface). Rats were then allowed to recover for 3 to 5 days. Sham-operated age-matched control rats underwent a similar procedure with manipulation of the left renal artery and implantation of intracerebroventricular cannula but without permanent application of a clip. At the end of the experiments, the position of the injection cannula was verified by the injection of blue ink (5 µL) and postmortem examination of the ventricular stain.

Procedure for AS-ODNs to AGT mRNA
To investigate the effects of AS-ODNs to AGT mRNA, thirteen 2K1C rats at 6 months after clipping were divided into 3 groups. An injection cannula (30 gauge), which was connected to a 10-µL Hamilton syringe by a 20-cm piece of PE-10 tubing, was inserted into the lateral ventricle, and AS (50 µg/5 µL, n = 5) or sense (50 µg/5 µL, n = 5) ODNs to AGT mRNA were injected for 1 minute. After 24 hours, we measured the SBP by the tail-cuff method. A blood sample was collected from the tail vein in 50 µL of 0.5 mol/L EDTA and 2.5 µL o-phenanthroline and centrifuged to collect plasma. Then the rats were deeply anesthetized, the brain was removed from the skull, and the hypothalamus and brain stem were separated. Ang II levels in plasma, hypothalamus, and brain stem were measured by radioimmunoassay. Plasma catecholamine concentrations were determined by high-performance liquid chromatography.

Procedure for AS-ODNs to AT1 R mRNA
We injected AS-ODNs to AT1 R mRNA (250 µg/kg per 5 µL, n = 6) or inverted ODNs (250 µg/kg per 5 µL, n = 5) into the lateral ventricle of the 2K1C rats 10 months after clipping. SBPs were measured by the tail-cuff method after 14 days. Fourteen days after intracerebroventricular injection of ODN, when SBP returned to the baseline, we injected AS-ODN-AT1 R (250 or 500 µg/kg per 250 µL, n = 5) intravenously through the tongue vein.

Antisense ODNs
AS, sense, or inverted ODNs were synthesized as 18-mers targeted to bases −5 to −11 of AT1 R mRNA and 15-mers to bases +77 of AT1 R mRNA.13 All the ODNs were phosphorothioated. The ODNs were dissolved in artificial cerebrospinal fluid (mmol/L: NaCl 137.0, KCl 3.0, MgCl2 1.3, NaHCO3 25.9, CaCl2 2.0, and glucose 10.0, pH 7.4) or saline for intracerebroventricular or intravenous injection, respectively, and stored at −20°C until use.

Ang II Assay
Plasma was frozen at −70°C until extraction with methanol on reversed-phase phenylisilylsilica extraction cartridges (Alpcor; approximate recovery, 90%). Samples were analyzed by double-antibody Ang II radioimmunoassay (RK-A22, Alpcor). The assay is sensitive to 0.7 pg/mL (0.7 pmol/L). Ang II levels were determined by a γ-counter (Beckman DP 550).

Statistical Analysis
Data are expressed as mean±SEM. Statistical analysis was performed by 1-way ANOVA, followed by the Fisher least significant difference method, with the use of Statview SE. A value of P < 0.05 was viewed as statistically significant.

Results

Effects of AS-ODNs to AGT mRNA
The baseline SBP was similar between the saline-treated, AS-ODN–treated, and sense ODN–treated groups (179±7, 207±13, and 177±10 mm Hg, respectively). Twenty-four hours after injection, SBP was significantly (P < 0.05) decreased in the AS-ODN–AGT–treated group compared with the control sense ODN–treated or saline-treated group (Figure 1A). Heart rate did not change in any group (Figure 1B). No significant differences in plasma Ang II were observed in 3 treatment groups, and plasma Ang II was significantly higher in aged-matched nonclipped rats (P < 0.05). However, the hypothalamic and brain stem Ang II levels in sense ODN–treated and saline-treated groups were significantly higher than those in age-matched nonclipped rats (P < 0.05),
and AS-ODN-AGT treatment significantly reduced the elevated hypothalamic Ang II levels to nonclipped concentrations ($P<0.05$), but no change in Ang II was found in the brain stem (Figure 2). Plasma levels of epinephrine were significantly lower in the AS-ODN–treated group than in the sense-treated group (37.0±6.69 compared with 482.7±90.9 pg/mL, respectively; $P<0.05$).

**Effects of AS-ODNs to AT1 R mRNA**

The baseline SBP was similar between the AS-treated and inverted ODN–treated groups (168±7 and 160±8 mm Hg, respectively). The time course of SBP after intracerebroventricular injection of ODN is shown Figure 3A. From 1 to 3 days after injection, SBP in the AS-ODN–treated group was significantly ($P<0.05$) lower than the baseline value. The decrease in SBP was also significant compared with the inverted ODN–treated group. Inverted ODN did not change SBP in the observation period. In contrast to the intracerebroventricular route, intravenous injection of AS-ODN-AT1 R did not affect SBP even at the higher dose (500 μg/kg) (Figure 3B).

**Discussion**

Chronic renovascular hypertension, lasting many months, is more clinically relevant than is acute renovascular hypertension, but few studies have investigated 2K1C hypertension many months after clipping. In the present study, we show that at 6 to 10 months after clipping of the renal artery, high BP persists even though plasma Ang II levels are no longer elevated. Indeed, the plasma Ang II levels were reduced in the chronic 2K1C rats. We hypothesized that the brain RAS is contributing to the maintenance of hypertension in chronic renovascular hypertension. The results show that brain RAS inhibition by AS-ODN-AGT and AS-ODN-AT1 R decreased SBP in the chronic phase of 2K1C rats. This inhibition was...
not due to leakage of AS-ODN out of the brain into the peripheral circulation, because intravenous injection of AS-ODN-AT,R at the same intracerebroventricular dose or at an even higher dose did not produce a change in BP. Therefore, we conclude that the lowering of BP in these rats by AS-ODNs reflected the role of brain RAS on the maintenance of BP in chronic renovascular hypertension.

Previously, it has been reported that in 2K1C rats, brain AGT mRNA is not different from that in normotensive rats. However, that was up to 4 months after clipping the renal artery. The present study shows significantly higher Ang II levels in the hypothalamus at 6 to 10 months after clipping. The hypothalamic Ang II level was significantly increased in 2K1C hypertensive rats compared with age-matched control rats, and this result is consistent with 1 other report. The levels of Ang II in the brain stem were also elevated in the chronic 2K1C rats, but only in the hypothalamus was the AS-ODN effective in reducing Ang II. This, the site of hypertensive action of AS-ODN appears to be the hypothalamus. Microinjection of AS-ODN-AT,R into the paraventricular hypothalamic nucleus (PVN) decreased the BP in Ren-2d gene transgenic rats. Injection of AS-ODN-AGT into the PVN of SHR attenuates vasopressin and catecholamine release. We have previously shown that the fluorescein isothiocyanate–labeled AS-ODN was taken up into the tissue around the third or lateral ventricle by intracerebroventricular injection and that AS-ODN-AT1 R at the same intracerebroventricular dose or at an even higher dose did not produce a change in BP. Therefore, we conclude that the lowering of BP in these rats by AS-ODNs reflected the role of brain RAS on the maintenance of BP in chronic renovascular hypertension.

Martin and Haywood have reported that stimulation of the PVN increases BP concomitant with an increase in plasma catecholamines, indicating activation of the sympathetic nervous system. The hypertension induced by a low dose of Ang II, which does not affect the plasma Ang II, is analogous to the chronic phase of renovascular hypertension. The centrally acting sympatholytic drug clonidine reverses the development of low-dose Ang II–induced hypertension. In the present study, AS-ODN-AGT decreased plasma epinephrine. Therefore, we propose that the mechanism of maintenance of high BP in chronic 2K1C hypertension involves high Ang II in the hypothalamus activating the PVN to increase sympathetic outflow, as shown by an elevation of circulating catecholamines.

The brain RAS may also be involved in the early stages of 2K1C hypertension, inasmuch as intracerebroventricular injection of Ang II antagonists such as saralasin can reduce BP. However, Sweet et al have reported that intracerebroventricular injection of a renin inhibitor or ACE inhibitor in 2K1C rats does not result in any change in SBP. This may indicate that an alternate pathway of Ang II synthesis was in action. AS-ODN-AGT decreased the elevated hypothalamic Ang II level in the present study. We speculate that the Ang II in the brain might be produced from AGT directly. The AS-ODN approach provides a specific and long-acting reduction in the brain RAS synthesis. A single injection of AS-ODN-AT,R reduced SBP for 3 days in the present study. The time course of the reduction of SBP seems to be characteristic of AS-ODN action and is similar to that which we previously reported in SHR. The largest reduction of BP was observed on the second day after injection in chronic 2K1C hypertensive rats and in SHR. From these and other studies, the inhibitory effects of AS-ODN take 24 hours to show a reduction of BP by inhibition of AT,R or AGT protein synthesis. Compared with a single dose of current pharmacological agents, the onset of the action of AS-ODN is slower, but the effect lasts much longer.

We showed that an intravenous injection of AS-ODN-AT,R in the same dose that was given intracerebroventricularly did not decrease SBP. We previously reported that the peripheral injection of liposome-encapsulated AS-ODN-AGT reduced BP in SHR. A liposome encapsulation increases uptake and decreases degradation of ODN. However, we did not use liposomes in the brain, because we have found that they are toxic in the central nervous system. Therefore, the intracerebroventricular injection of AS-ODN was without liposomes, we did not use AS-ODN with liposomes for the control intravenous injection. This did not preclude the AS-ODN from having an effect, inasmuch as peripheral injection of AS-ODN-AT,R decreased SBP in cold-induced hypertension without liposome encapsulation. Thus, when the peripheral RAS is activated, as in cold-induced hypertension, AS-ODN inhibition of RAS can reduce BP. The dose of injection in a previous study was similar to that of the lower dose in the present study. Because we could not observe a reduction of BP in the chronic 2K1C rat with an intravenous injection of AS-ODN even at the higher dose, we conclude that peripheral RAS does not have an important role in the maintenance of BP in the chronic phase of renovascular hypertension.

We studied the chronic phase in 2K1C hypertensive rats because human patients presenting with renovascular hypertension have generally had the disease for a long time. Although there is no direct evidence that the brain RAS contributes to the maintenance of high BP in human renovascular hypertension, an overactivation of the sympathetic nervous system is observed, and centrally acting drugs such as clonidine have been shown to reduce BP in chronic unilateral renal artery stenosis. Therefore, the results are relevant to understanding the mechanisms of chronic renovascular hypertension, and we conclude that the brain RAS plays a significant role by elevating sympathetic outflow from the brain. The results also point to a therapy strategy that could be developed to treat this form of hypertension.

Acknowledgments

This work was supported by National Institutes of Health MERIT award HL-27334. Dr Kagiyama is supported by the Japan Heart Foundation and a Bayer Yakuhin Research Grant Abroad.

References

1. DeForrest JM, Knappenberger RC, Antonaccio MJ, Ferrone RA, Creekmore JS. Angiotensin II is a necessary component for the devel-


Antisense Inhibition of Brain Renin-Angiotensin System Decreased Blood Pressure in Chronic 2-Kidney, 1 Clip Hypertensive Rats
Shuntaro Kagiyama, Adrian Varela, M. Ian Phillips and Sara M. Galli

Hypertension. 2001;37:371-375
doi: 10.1161/01.HYP.37.2.371

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/37/2/371

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/