Angiotensin II AT₁A Receptor Antisense Lowers Blood Pressure in Acute 2-Kidney, 1-Clip Hypertension

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Abstract—To test the effectiveness of antisense oligonucleotides targeted to the angiotensin type 1A (AT₁A) receptor mRNA on blood pressure reduction, the 2-kidney, 1-clip (2K1C) Goldblatt model of hypertension was studied in the acute phase of hypertension, when the peripheral renin-angiotensin system is overactive. A single injection of AT₁A receptor antisense oligodeoxynucleotides significantly reduced systolic blood pressure for a period of 8 days in 2K1C rats after clipping, from 157.5±5 mm Hg on day 7 to 141.3±3.0 mm Hg on day 15 after clipping (P<0.01). The AT₁A receptor antisense oligonucleotide labeled with fluorescein shows that the antisense oligonucleotide at 24 hours was taken up into aorta, mesenteric artery, liver, kidney glomeruli, and medulla, remaining up to 6 days. The AT₁A receptor number in fmol/g tissue was significantly decreased after AT₁A receptor antisense oligonucleotide treatment in the dorsal aorta, mesenteric artery, renal cortex, and renal medulla (P<0.05) compared with that of the AT₁A receptor–scrambled antisense oligonucleotide control-treated group. The data clearly demonstrate a prolonged antihypertensive effect of AT₁A receptor antisense oligonucleotide in the 2K1C renovascular model of hypertension when it is administered intravenously in a single low dose (0.33 mg/kg²). It also shows that the AT₁A receptor antisense oligonucleotide is actively taken up by AT₁A target tissues and that there is a significant decrease in receptor density. We conclude that in the acute phase of 2K1C hypertension, antisense to AT₁A receptor decreases AT₁A receptor density, which attenuates the vascular vasoconstrictive effects of high plasma angiotensin II levels and in the kidney elicits natriuresis. The decrease in renal AT₁A receptor density may also lead to sodium loss and reduction of extracellular volume. (Hypertension. 2001;38[part 2]:674-678.)

Key Words: hypertension, 2K1C oligonucleotides, antisense AT₁A receptors angiotensin II

Angiotensin (Ang) II is a potent vasoconstrictor peptide, produced by the renin-angiotensin system (RAS). The widespread use of ACE inhibitors ACE, and more recently Ang II receptor blockers in the treatment of human hypertension, point to the importance of reducing overactive RAS genes to lower blood pressure (BP). The effects of Ang II are mediated through plasma membrane receptors, one of which is the angiotensin type 1 receptor (AT₁R). The AT₁R is responsible for the majority of cardiovascular effects associated with Ang II. As a prelude to a gene therapy approach to hypertension, we have made antisense oligodeoxynucleotides (AS-ODNs) targeted to the sequence of the AT₁R for in vivo use in reducing hypertension. AS-ODN, or antisense DNA in vectors, produces inhibition of specific protein synthesis because of its unique specificity. In the case of the AT₁R, there are 2 subtypes, AT₁A and AT₁B; both are found in rats, but only AT₁A in humans. The design of our AS-ODN is specifically targeted to the AT₁A sequence. The technique of antisense inhibition offers not only specificity but also prolonged reductions in BP for several days with a single dose. So far, we have established this depressor action with AS-ODNs in spontaneously hypertensive rats and in a model of environmentally induced hypertension, cold-induced hypertension. In both models, in vivo application of antisense targeted to AT₁R or to angiotensinogen production, the AS-ODN reduced BP by 20 to 30 mm Hg. The hypothesis for the present study is that AT₁A-AS-ODN will decrease the hypertensive effect of an overactive RAS. To test the hypothesis, we studied the 2-kidney, 1-clip (2K1C) acute phase of hypertension, the BP response and the tissue AT₁R density and tissue ODN uptake after AT₁A-AS-ODN treatment compared with those of the 2K1C control group treated with AT₁A-scrambled oligonucleotide (AT₁A-Scr-ODN).

Methods

Animals

Adult male Sprague Dawley rats (250 to 280 g), acquired from Harlan (Indianapolis, Ind), were kept in individual cases in a room on a 12-hour/12-hour light/dark cycle and on tap water and standard rat chow at libitum. During the first week of adaptation period, systolic BP (SBP) was recorded between 9:00 and 11:00 AM 3 times per week to assess SBP basal levels. The left renal artery was clipped on the following week. This procedure was performed under anesthesia (mixture of 35 mg/kg ketamine and 7 mg/kg xylene SC). A silver clip of 200 µm was placed into the left renal artery via a dorsolateral incision.
approach. The animals were left to recover for 3 days. In the AT$_{1A}$-R-AS-ODN group, the SBP was measured by tail-cuff method a week before clipping for baseline values and up to day 25 after clipping as explained below.

Methods

Oligodeoxynucleotides

The ODNs were synthesized as 15-mer phosphorothioated ODNs, following the procedure described earlier. The sequence homology was tested against all available DNA sequences in the GenBank for uniqueness. The purity of each batch of ODNs was tested with high-performance liquid chromatography. ODNs were dissolved in 0.9% sterile saline solution, aliquotted in a concentration of 50 $\mu$g/mL, and stored at $-20^\circ$C.

BP Response to a Single Intravenous Dose of AT$_{1A}$-R-AS-ODN

For SBP baseline values, BP was measured 3 times a week before clipping. On day 7 after clipping, when SBP reached a steady level of 156 to 160 mm Hg, the rats were divided into 2 groups: (1) In the control group, the 2K1C rats (n=6) were treated with AT$_{1A}$-R-Scr-ODN (0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV), and the SBP was recorded daily from day 8 to 16, ending on day 16 after clipping. (2) In the antisense group, the rats (n=5) were injected with a single injection of AT$_{1A}$-R-AS-ODN (0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV). SBP was recorded daily from day 5 to day 10 after clipping and thereafter on days 13, 15, 21, and 25 after clipping. The ODNs were dissolved in sterile 0.9% saline solution and injected into the tail vein in a volume of 100 to 200 $\mu$L.

Tissue Distribution of Fluorescein-Labeled AS-ODN by Laser Scanning Confocal Microscopy

AT$_{1A}$R-AS-ODN and AT$_{1A}$R-Scr-ODN labeled on 5’ and 3’ ends with fluorescein isothiocyanate (FITC) were administered in a single dose (0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV) in 2K1C rats (n=8) for each group on the day 7 after clipping. Two animals per group were killed at 24, 48, 120, and 144 hours after FITC-ODNs injection and perfused transcardially with 30 mL isotonic heparinized (0.5%) PBS (pH 7.4) followed by 50 mL 4% paraformaldehyde in 0.9% isotonic PBS solution (pH 7.4). The liver, aorta, and mesenteric arteries were removed, kept in 4% paraformaldehyde in PBS for 24 hours and then in a 30% sucrose PBS solution (pH 7.4), and finally sectioned and mounted on slides for laser confocal examination of the FITC-ODN fluorescence uptake.

Determination of Tissue AT$_{1R}$ Density by Quantitative Receptor Autoradiography

On day 7 after clipping, a group of 2K1C rats (n=5) was injected with a single dose of AT$_{1A}$-R-AS-ODN (0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV), and another group of n=5 was injected intravenously with the same dose of AT$_{1A}$-R-Scr-ODN. Forty-eight hours after ODNI administration, the animals were deeply anesthetized with ketamine-xylene mix subcutaneously and transcardially perfused with heparinized PBS (pH 7.4). Thoracic aorta, mesenteric artery (at 5 mm from abdominal aorta), and kidneys were quickly removed, immediately frozen on dry ice, and kept at $-70^\circ$C. Six to 10 cryostat sections of each tissue were cut at 20-μm thickness. Tissue autoradiography for Ang II-binding was performed as described previously. For the microdensitometric quantification of the autoradiograms, measurements of 5 to 7 areas of each section were taken, and average value was obtained for each of the incubated sections. The amount of $^{125}$I-Sar$^1$, Ile$^8$-Ang II bound is expressed in fmol/g wet tissue.

Test to Determine Natriuretic Effect of AT$_{1A}$-R-AS-ODN Treatment in Control Unclipped Rats

To simply test the direct effect of AT$_{1A}$-R-AS-ODN and AT$_{1A}$-R-Scr-ODN on renal sodium excretion, ODNs were administered intravenously directly into the left renal artery of normotensive Sprague-Dawley males (n=4 each group). In each group, urine was collected at 24 hours (n=2) and 48 hours (n=2). Kidneys were taken at 24 to 48 hours for autoradiography to determine AT$_{1A}$-specific binding in both kidneys. To enhance ODN uptake by the renal tissue, ODNs were administered on a solution of 1:3 ratio with liposomes.

Statistical Procedures

All numerical values were expressed as mean±SEM. Statistical analysis was performed using Sigmasstat (Jandel Scientific) computer software. One-way ANOVA was used to determine treatment effect in the receptor-binding studies, whereas 2-way ANOVA was used to analyze BP data. Duncan multiple range test was used for individual comparisons. $P<0.05$ was considered statistically significant.

Results

BP Response to a Single Injection of AT$_{1A}$R-AS-ODN in the Early Phase of Hypertension

Hypertension (156 to 160 mm Hg) was observed on day 7 to 8 after clipping (Figure 1). SBP was significantly elevated after day 5 after clipping compared with SBP basal levels before left renal artery clipping ($P<0.05$). AT$_{1A}$-R-AS-ODN significantly reduced SBP (30±4 mm Hg; $P<0.01$). This reduction lasted at least 8 days, following a single intravenous ODN injection of a dose of 0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV (Figure 1, day 15 after clipping). The control group (treated with 0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV of AT$_{1A}$R-Scr-ODN) developed hypertension >150 mm Hg from day 8 after clipping. Heart rate was not changed after ODN treatment in both group studied.

AS-ODN Tissue Uptake

The administration of FITC-AT$_{1A}$-ODNs (0.33 mg · kg$^{-1}$ · g$^{-1}$ body weight IV) into 2K1C at 7 days after clipping showed that the AT$_{1A}$-R-AS-ODN and AT$_{1A}$-R-Scr-ODN fluorescence is clearly taken up by mesenteric artery, kidney, tissue, and liver. Fluorescent micrographs reveal significant tissue uptake with intensity diminishing in a time-course–dependent manner. At 24 hours after injection, FITC-ODNs are taken up very efficiently by hepatocytes with a predominantly cytos-
plasmic distribution. By day 6 after injection, FITC content in hepatocytes had diminished dramatically, although some diffuse cytoplasmic signal can still be observed. The efficiency of the AT\textsubscript{1A}R-AS-ODN uptake by the nonclipped kidney proximal tubular cells and vasculature of 2K1C is confirmed in our studies by the intense uptake of the AT\textsubscript{1A}R-AS-ODN labeled with FITC (Figure 2, bottom). One day after FITC-ODN injection, the clipped and nonclipped kidneys both appear to have picked up substantial amounts of the FITC-ODN molecule, with much of the tag localized to the cytoplasm of the proximal tubular epithelial cells and little, if any, in distal tubular cells. FITC-AT\textsubscript{1A}R-AS-ODN in clipped and nonclipped kidneys remains relatively high 6 days after injection (Figure 2, 24 hours and 6 days, nonclipped kidneys). FITC-ODN uptake in both the aorta and mesenteric artery at 1 day after injection was quite substantial and was found in smooth muscle cells, in intercellular spaces surrounding smooth muscle, and in endothelial cells (Figure 2, top). Significant amount of FITC-AT\textsubscript{1A}R-AS-ODN in the aorta and mesenteric artery was retained at 6 days after injection, similar to the amount found in the kidneys.

**Ang II AT\textsubscript{1A}R Response to AT\textsubscript{1A}R-AS-ODN Administration**

After 24 hours of intravenous administration of 0.33 mg · kg\textsuperscript{-1} · g\textsuperscript{-1} body weight IV, the autoradiographic studies showed a significant decrease in the specific binding of the labeled Ang II by the AT\textsubscript{1A}R, indicating a significant decrease in the total number of AT\textsubscript{1A}Rs by the thoracic dorsal aorta, mesenteric artery, and renal tissue compared with that of the AT\textsubscript{1A}-Scr-ODN–treated group (\(P<0.05\)). In the mesenteric artery (Figure 3B), and to a lesser extent in the thoracic dorsal aorta (Figure 3A), a significant decrease in the number of AT\textsubscript{1A}Rs was found. In both kidneys, AT\textsubscript{1A}R-AS-ODN was equally effective in reducing the Ang II AT\textsubscript{1A}Rs in the renal cortex and renal medulla (Figure 3C, nonclipped kidney). The magnitude of the decrease in Ang II AT\textsubscript{1A}R binding is larger in the renal medulla than in the renal cortex.
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Figure 4. Autoradiograms of labeled Ang II binding showing specific AT1R density in the renal cortex and renal medulla. A, Normotensive untreated control Sprague Dawley rat. B and C, Autoradiograms of nonclipped kidney of the 2K1C-treated rats with ODN at 24 hours. B, 2K1C injected intravenously with 0.33 mg · kg⁻¹ · g⁻¹ body weight of AT1A-Scr-ODN. C, 2K1C rats injected intravenously with 0.33 mg · kg⁻¹ · g⁻¹ body weight of AT1A-R-AS-ODN. On the right, standard-label Ang II–binding reading scale in fmol/g tissue.

receptors appear to be located in interstitial cells, parallel to the vasa recta. For the 2K1C study described above, autoradiograms showing the nonclipped kidney renal cortex and medulla AT1R distribution are presented in Figure 4 and compared with a normal nonclipped control rat kidney (Figure 4A). Figure 4B shows the nonclipped kidney of 2K1C rat treated with AT1A-Scr-ODN. The nonclipped kidney of a rat treated with AT1A-R-AS-ODN is shown in Figure 4C. From the autoradiograms, it is clearly observed that AT1A-R-AS-ODN in the nonclipped kidney significantly decreases AT1R binding, both in the renal cortex and renal medulla, compared with that of the Scr-ODN control group.

Urinary Sodium Excretion

In the normotensive rats, the direct renal artery administration of AT1A-R-AS-ODN at 24 hours, increased urinary sodium (U₉₉) excretion from pre-ODN value of 0.186 to 0.734 (μEq/min⁻¹/100 g body weight; n = 2). At 48 hours, U₉₉ excretion changed from 0.137 to 0.261 (μEq/min⁻¹/100 g body weight; n = 2). The AT1A-Scr-ODN treatment did not cause significant changes in sodium excretion either at 24 hours (from 0.113 to 0.135 μEq/min⁻¹/100 g body weight) or at 48 hours (from 0.131 to 0.102 μEq · min⁻¹/100 g body weight). On a second experiment, the same natriuretic response to AT1A-R-AS-ODN treatment was observed. Because of large dispersion of baseline values, data are not included.

The autoradiography for AT1R binding in this study shows a number of adrenal medulla AT1 receptors, equal to 1800 ± 250 fmol/g tissue, for the left kidney injected with AT1A-Scr-ODN at 24 hours. In the left kidney treated with AT1A-R-AS-ODN, the AT1R number decreased to a low level of 100 ± 25 fmol/g tissue. The renal cortex AT1R number also changed significantly, from 1400 ± 141 to 260 ± 70 fmol/g tissue. The right kidney showed a similar pattern of response for renal medulla and renal cortex, but the decrease in AT1R number after ODN treatment was to a lesser extent.

Discussion

The results show that during the early phase of hypertension in the 2K1C rat model, a single injection of a low dose of AT1A-R-AS-ODN (0.33 mg · kg⁻¹ · g⁻¹ body weight), targeted to AT1R mRNA, significantly decreases BP (P < 0.01). The control AT1A-Scr-ODN tested in this model showed no effect on BP. The decrease in BP lasts for 8 days before returning to pretreatment SBP levels. No change in heart rate was detected. The results clearly demonstrate that AT1A-R-AS-ODN is effective in lowering SBP in the 2K1C model of renovascular hypertension. In chronic 2K1C rats at 6 and 10 months after clipping, AT1A-R-AS-ODN was not effective intravenously, but it was effective when injected into the brain. We interpret this as meaning that in the acute phase, 2K1C is plasma renin dependent, but in the chronic phase it is brain RAS dependent. AT1A-R-AS-ODN was also effective in reducing BP in spontaneously hypertensive rats and cold-induced hypertension. The question of whether liposomes or carriers are necessary was addressed in another study. Delivery of the ODN as AS-ODNs liposome mix in 2K1C rats at 30 days after renal artery clipping effectively reduced BP for >10 days.

In our studies of the 2K1C, SBP response to AT1A-R-AS-ODN, the reduction in AT1A-R protein is evidenced by the differential changes in Ang II AT1R numbers compared with that of AT1A-Scr-ODN–treated animals, shown by the quantitative receptor autoradiography. In the thoracic aorta, there was a significant decrease in Ang II AT1R binding. In mesenteric artery, the AT1R binding was significantly reduced, at a greater extent than in the dorsal aorta. The importance of the AT1A R on vascular resistance is further supported by studies on AT1A knockout mice. The disruption of AT1A R in mice decreases SBP in 24 mm Hg, and the pressor response to infused Ang II is virtually absent. Another study on AT1A knockout mice shows attenuation of renal vasoconstriction to Ang II. The inhibition of this response by AT1R antagonist in mice suggests renal vasoconstrictive mediation by AT1R. In the 2K1C rat model, the distribution and role of AT1Rs is not well known. In the 2K1C acute phase treated with AT1A-R-AS-ODN, AT1Rs may be

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activated but not to the extent to reverse the hypotensive response elicited by the significant decrease in vascular and renal AT\(_1\)Rs. It has been suggested a role for AT\(_1\)Rs in normalized SBP in conditions in which AT\(_1\)Rs are blocked by specific antagonist.\(^6\) In the 2K1C study, we did not observe a significant number of vascular AT\(_1\)Rs or a difference between the ODN-treated groups. Further studies are necessary to determine the possibly vasodilatory action of AT\(_1\)R in 2K1C treated with AT\(_1\)A-AS-ODN.

In our study, reductions of AT\(_1\)R density were also seen in the kidney where the AT\(_1\)R can influence both sodium reabsorption and glomerular vascular resistance. In the 2K1C, both cortex and medulla AT\(_1\)R density were affected by the AT\(_1\)A-AS-ODN, but the larger decrease in AT\(_1\)R density was observed in the renal medulla. The decrease in AT\(_1\)Rs found in the renal cortex corresponds to the glomerular receptors. The decrease in afferent arteriole and mesangial cells AT\(_1\)Rs may lead to increased glomerular filtration rate and urine volume. Proximal tubular AT\(_1\)Rs modulate the amount of sodium reabsorbed.\(^7\)\(^–\)\(^9\) In the 2K1C rats, the blockade of the RAS has been shown to cause increase in urine flow and \(U_{\text{Na}}\) excretion.\(^10\)\(^–\)\(^22\) The proximal tubular epithelial cells of the 2K1C kidney retain the labeled AT\(_1\)-AS-ODN for a long period of time, suggesting that the decrease in AT\(_1\)R may last for days, affecting the total amount of sodium filtered and reabsorbed by the kidney. In this model of hypertension, the elimination of more sodium and water in the urine would lead to a reduction of extracellular fluid and indirectly contribute to ameliorate the hypertension.\(^23\) The medulla AT\(_1\)R may also indirectly influence the total amount of sodium and water excreted by the kidneys.\(^24\) In the normotensive control study, the kidney autoradiography at 24 hours showed a significant decrease in AT\(_1\)R density in renal cortex and medulla. One of the reasons for the magnified decrease in kidney AT\(_1\)R density in this group, may be explained by the local injection, on site, of the ODN directly into the renal artery. Another reason may relate to the ODN-liposome mix administered. The dramatic decrease in AT\(_1\)R in the AT\(_1\)A-AS-ODN–treated unclipped rats may explain the significant natriuretic response observed in this group.

In conclusion, the present study shows that the AS-ODN targeted to the AT\(_1\)A-R was effective in reducing high BP in the acute phase in the 2K1C model of renal vascular hypertension. The mechanism of action appears to be due to the uptake of AT\(_1\)A-AS-ODN into blood vessels, kidney tubules, and glomeruli, where the oligonucleotide specifically inhibited AT\(_1\)R synthesis, reducing effectively, the number of AT\(_1\)Rs available. This reduction in AT\(_1\)Rs may be the major cause for the hypotensive response in the presence of high plasma Ang II. It may also contribute to reduce volume overload by its direct action on the kidneys.

References


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