Angiotensinogen Gene–Activating Elements Regulate Blood Pressure in the Brain

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Abstract—Although the angiotensinogen gene is a possible candidate as a determinant of hypertension, the molecular mechanisms of tissue angiotensinogen gene regulation have yet to be clarified. We identified essential transcription regulators of angiotensinogen production in the central nervous system using synthetic double-stranded oligodeoxynucleotides (ODNs) as “decoy” cis elements to block the binding of nuclear factors to promoter regions of the targeted gene. Using a gel mobility shift assay, angiotensinogen gene–activating element (AGE) 2 binding protein was detected in the brain nuclear extracts of both spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats (WKYs). Importantly, the binding activity of AGE 2 and angiotensinogen mRNA level were significantly higher in the brain of SHRs than in that of WKYs. Using the decoy approach, we demonstrated a significant decrease in the blood pressure of SHRs by transfection of AGE 2 decoy, but not mismatched, ODNs into the lateral cerebroventricle, accompanied by a significant decrease in brain angiotensinogen concentration and mRNA, and angiotensin II level. That these effects, demonstrated herein, are due to central effects is confirmed by the fact that no changes in circulating levels of angiotensinogen or angiotensin II concentrations were observed. Notably, AGE 2 decoy ODNs did not decrease the blood pressure of WKYs. We conclude that the abnormal expression of AGE 2 binding protein in the central nervous system plays a crucial role in high blood pressure of a genetically hypertensive rat model. (Circ Res. 1999;85:257-263.)

Key Words: transcriptional cis element ■ central nervous system ■ HVJ-liposome method ■ renin-angiotensin system ■ decoy oligodeoxynucleotide

Angiotensinogen, which is mainly produced in the liver, plays important roles in the renin-angiotensin system in the pathogenesis of essential hypertension. Results of recent genetic studies suggest that the angiotensinogen gene is a possible determinant of hypertension.1–4 The role of angiotensinogen in the regulation of blood pressure was previously demonstrated by the observation that anti-angiotensinogen antibody administration resulted in a reduction in blood pressure.5 In contrast, in another study acute administration of pure rat angiotensinogen to rats resulted in an increase in blood pressure.6 Using a “loss-of-function” approach, we and others have reported that generation of circulating angiotensinogen is a rate-limiting step in blood pressure regulation.7–9 Given the importance of angiotensinogen regulation in the pathogenesis of hypertension, understanding how the angiotensinogen gene itself is regulated is essential. One study suggests that the angiotensinogen gene is regulated by novel transcriptional factors such as angiotensinogen gene–activating factor (AGF) 1 to 3 in cultured human hepatocytes (HepG2 cells) in vitro.10 Previously, we demonstrated that angiotensinogen gene–activating element 2 (AGE 2), which is the proximal promoter element extending from –96 to –52, has a pivotal role in the regulation of hepatic angiotensinogen production in vivo.11

On the other hand, accumulating evidence has indicated the presence of a tissue renin-angiotensin system that involves the central nervous system.12–15 However, the molecular mechanisms of angiotensinogen gene regulation in the brain remain unknown. Moreover, no reports have described the importance of brain angiotensinogen in blood pressure control. Therefore, in this study, we examined (1) how brain angiotensinogen is regulated in vivo and (2) whether brain angiotensinogen regulates blood pressure. To answer these questions, we used synthetic double-stranded oligodeoxynucleotides (ODNs) as “decoy” cis elements to block the binding of nuclear factors to promoter regions of the targeted gene, resulting in the inhibition of gene transactivation16–19 (Figure 1).

Materials and Methods

Synthesis of ODNs and Selection of Sequence Targets

The sequences of phosphorothioate double-stranded ODNs complementary to AGF 2 and AGF 3 binding sites and mismatched ODNs...
residue was hydrated in 200 mM Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA-2Na (1 mg/mL whole blood) and 2.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)) and 25% glycerol) with one change after 2 hours. After centrifugation at 12 000 g (Pharmacia Biotech), MgCl2 1.5, KCl 10, and DTT 0.5. After centrifugation at 12 000g for 15 minutes at 4°C, each pellet was lysed in 1 volume of ice-cold homogenization buffer (in mmol/L, NaCl 137, KCl 3, Na2HPO4 8, and KH2PO4 1), and immediately frozen in liquid nitrogen for use in the gel mobility shift assay and measurement of brain angiotensinogen and angiotensin (Ang) II concentrations. All brain tissue was stored at –80°C before use. Blood samples were also collected in prechilled tubes containing EDTA-2Na (1 mg/mL whole blood) and 2.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 4°C. To measure Ang II, plasma was stored at –80°C before assay.

**Gel Mobility Shift Assay**

Nuclear extracts were prepared from transfected brain as described previously. In brief, rat brains were homogenized in a Wheaton all-glass Dounce homogenizer in 2 volumes of ice-cold homogenization buffer (in mmol/L, HEPES [pH 7.9] 10, PMSF 0.5, MgCl2 1.5, KCl 10, and DTT 0.5). After centrifugation at 12 000g for 15 minutes at 4°C, each pellet was lysed in 1 volume of ice-cold homogenization buffer (in mmol/L, PMSF 0.5, HEPES [pH 7.9] 20, MgCl2 1.5, DTT 0.5, and EDTA 0.2; 0.1 mol/L KCl; and 20% [vol/vol] glycerol) by homogenization in the Dounce homogenizer. The supernatant was purified using a NICK column (Pharmacia) and quantified with a spectrophotometer. ODNs were annealed for 40 minutes while the temperature decreased from 85°C to 25°C.

**Preparation of HVJ–Liposome Complex**

Hemagglutinating virus of Japan (HVJ, Z strain) was propagated in chorioallantonic fluid of embryonated eggs, as previously described. HVJ was collected by centrifugation at 27 000 g, the ODN liposomes were incubated at 37°C for 30 minutes at 4°C, and the supernatant was determined. Aliquots containing protein were snap frozen in liquid nitrogen and stored at –80°C. Decoy ODNs were labeled as probes at the 5′ end using a 5′ end-labeling kit (KinaseMax, Ambion). Binding reactions containing nuclear extract (50 µg), poly(dI-dC) (2 µg) (Pharmacia Biotech), 10× binding reaction buffer (40% glycerol and [in mmol/L], EDTA 10, b-mercaptoethanol 10, Tris-HCl (pH 7.5) 100, and NaCl 400) and 32P-labeled ODNs (~10 000 cpm) were incubated for 30 minutes at room temperature and then loaded onto a 5% PAGE and dried. For the control, samples were incubated with an excess (2×, 4×) of unlabeled AGE 2 and 3 ODNs, which completely abolished binding.
Gels were analyzed by autoradiography. The activity of binding protein was quantified and compared by densitometric analysis.

**Rat Angiotensinogen and Ang II Assay**

The brain or plasma angiotensinogen level was determined indirectly by the measurement of Ang I generated after incubation with excess recombinant human active renin (kindly donated by Dr Fumiaki Suzuki, Gifu University, Japan), as described previously.22,23 Samples were homogenized using a Polytron in angiotensinogen RIA buffer (in mmol/L, Na2HPO4 150, NaCl 160, and EDTA 3, and 5% BSA [pH 6.5]) and centrifuged at 12 000g for 30 minutes at 4°C. The supernatant or 1-mL sample of plasma was incubated with 1 mL (Goldblatt units) recombinant human active renin and 1 mmol/L PMSF for 2 hours at 37°C. During the incubation, angiotensinogen was completely converted to Ang I.22,23 The Ang I level of tissue sample was determined by RIA, and the results were expressed as nanograms of Ang I per gram tissue.

To measure Ang II in the brain samples, the brain was homogenized using a Polytron in 0.1N HCl (2 mL/g tissue), boiled in a waterbath for 7 minutes, and centrifuged at 12 000g for 30 minutes at 4°C. The supernatant was applied to an octyl minicolumn (Amprep C8, Amersham), which had been prewashed with 3 mL of 100% methanol and 3 mL of 0.1% trifluoroacetic acid (TFA).24,25 After washing the column with 3 mL of 0.1% of TFA, Ang II was eluted with 2 mL of ethanol:water:TFA (80:19.9:0.1, vol/vol). The eluant was dried under vacuum in a centrifugal concentrator (CC-181, Tommy) and the resultant Ang II– containing residue resuspended in 100 µL of 0.1% TFA. HPLC characterization was then performed as previously described.24,25 In the appropriate peak fractions, samples were collected and dried in a vacuum centrifuge and dissolved in 0.1 mol/L Tris-acetate, pH 7.4, containing 2.6 mmol/L EDTA-2Na, 1 mmol/L PMSF, and 0.1% BSA. The immunoreactive Ang II was measured using RIA with a specific Ang II antibody. A 1-mL sample of each plasma specimen was promptly concentrated in an Amprep C8 minicolumn and quantified using the same procedure.24,25 The recovery of Ang II was 98 ± 5%. The cross-reactivity of the Ang II antibody was 100% for Ang II and <0.1% for Ang I, Ang III, or Ang-(1–7).

**RNA Analysis**

For Northern blot analysis, the brain was promptly removed from rats before transfection (SHRs, n = 5; WKYs, n = 5), and SHRs were transfected with decoy ODNs 3 days after transfection (AGE 2 decoy ODNs, n = 5; mismatch decoy ODNs, n = 5). All brain tissues were frozen in liquid nitrogen and stored at −80°C before RNA extraction. The whole brain (except cerebellum) was homogenized with RNAzol B (2 mL/100 mg tissue, Tel-Test, Inc) using a Polytron, chloroform added to extract the RNA, and the mix centrifuged at 12 000g for 15 minutes at 4°C. The aqueous phase was collected, and isopropanol was added to precipitate the RNA. After centrifugation, the RNA pellet was washed in 75% ethanol and dissolved in Tris-EDTA. For Northern blot analysis, 20 μg of total RNA was electrophoresed through a 1.5% agarose-formaldehyde denaturing gel and transferred to nitrocellulose membrane (Amersham International). The filter was baked, prehybridized, and hybridized to full-length cDNA for rat angiotensinogen22,23 and human GAPDH cDNA control probe (Clontech Laboratories, Inc); both were labeled with 32P, with the Rad Prime DNA Labeling System (Life Technologies, Inc), for Northern blotting. The filter was then washed and exposed to x-ray film. The expression of mRNA was quantified and compared by densitometric analysis.

**Statistical Analysis**

The results are expressed as mean ± SEM. Statistical analysis was performed using ANOVA followed by multiple comparisons. When appropriate, the repeated measures option was applied to the ANOVA. The Duncan multiple range test was applied to assess differences whenever statistical significance was found. The Student t test was also used for paired and unpaired observations. For statistical purposes, values below the detection levels of an assay were recorded as the detectable level. Statistical significance was established at the P < 0.05 level.

**Results**

**Role of AGE 2 Binding Protein in Brain Angiotensinogen Production and High Blood Pressure Regulation in SHRs but Not WKYs**

To detect AGE binding proteins, gel mobility shift assay was performed using nuclear extracts and synthetic double-stranded ODNs that had sequence homology with AGE 2. Similar to the liver,10,11 AGE 2 binding protein was detected in brain tissue (Figure 2A and 2B). Moreover, complete competition for the increased binding of AGE 2 by an excess amount of AGE 2 decoy ODNs, but not mismatched AGE 2 decoy ODNs, was observed (Figure 2A), which was consis-
tent with a previous report.\textsuperscript{10,11} Importantly, the binding activity of AGE 2 was significantly higher in SHRs than WKYs ($P < 0.01$, Figure 2B and 2C).

Given the significant activation of AGE 2 binding protein, we hypothesized that the activation of AGE 2 binding activity may contribute to higher upregulation of angiotensinogen gene expression in SHRs than WKYs, thereby resulting in the high blood pressure of SHRs. Indeed, the mRNA level of angiotensinogen was significantly higher in the brains of SHRs than WKYs ($P < 0.05$, Figure 3). Therefore, we examined the inhibitory effects of decoy ODNs against AGE 2 on the high blood pressure of SHRs. Because we previously reported the highly efficient ODNs transfection into the brain using the HVJ-liposome method,\textsuperscript{26} we first examined the physiological effect of AGE 2 decoy ODN transfection by the HVJ-liposome method into the lateral cerebroventricle. As shown in Figure 4A, transfection of AGE 2 decoy ODNs (20 \textmu m/L) into the intracerebroventricle resulted in a significant transient decrease in systolic blood pressure in SHRs at 1 day after transfection through 7 days. On the other hand, transfection of mismatched decoy ODNs did not change blood pressure. Decrease in systolic blood pressure observed in AGE 2 decoy ODN-transfected rats reached statistical significance at 1, 2, 4, and 7 days after transfection as compared with blood pressure in mismatched decoy ODN-transfected rats. No changes in systolic blood pressure were observed in response to either transfection of AGE 2 decoy ODNs or mismatched decoy ODNs in WKYs (data not shown). As shown in Figure 4B, a lower concentration of ODNs (5 \textmu m/L) did not decrease blood pressure in SHRs (mismatched decoy ODNs, 229±3 mm Hg; 5 \textmu m/L of AGE 2 decoy ODNs, 225±4 mm Hg; NS) and a higher concentration of ODNs (20 and 40 \textmu m/L) elicited significant decrease in systolic blood pressure in SHRs (20 \textmu m/L, 213±6 mm Hg; 40 \textmu m/L, 201±4 mm Hg; $P < 0.05$ versus mismatched). The significant contribution of AGE 2 to high blood pressure in SHRs was also supported by the observation that transfection of AGE 2 decoy ODNs did not decrease blood pressure in WKYs, unlike in SHRs. This observation was further confirmed by the fact that there was no significant change in brain angiotensinogen and brain Ang II concentrations in WKYs transfected with AGE 2 decoy ODNs (data not shown). Plasma concentrations of either angiotensinogen or Ang II showed comparable levels in SHRs or WKYs between decoy-transfected and control animals (data not shown).

**Role of AGE 3 Binding Protein**

As mentioned above, AGE 3 (from −6 to +22), which is located directly around the transcriptional start site in the core
promoter region, may also regulate angiotensinogen production. Similar to AGE 2 binding protein, AGE 3 binding protein was also detected in the brain tissue (Figure 7A and 7B). Moreover, complete competition for the increased binding of AGE 3 by an excess amount of AGE 3 decoy ODNs, but not mismatched AGE 3 decoy ODNs, was observed (Figure 7A), consistent with a previous report.10,11 The binding activity of AGE 3 was also significantly higher in SHRs than in WKYs (P < 0.01, Figure 7B and 7C). However, transfection of AGE 3 decoy ODNs into the brain did not induce any significant change in high blood pressure in SHRs (data not shown). Brain angiotensinogen and Ang II concentrations were also not significantly different between SHRs transfected with AGE 3 decoy ODNs and those transfected with mismatched AGE 3 decoy ODNs (data not shown). Similar to WKYs, transfection of AGE 3 decoy ODNs into the brain failed to induce a change in blood pressure. There were no significant differences in brain angiotensinogen and Ang II concentrations between WKYs transfected with AGE 3 decoy ODNs and those transfected with mismatched AGE 3 decoy ODNs (data not shown).

Discussion

Angiotensinogen is the precursor of the vasoactive peptide Ang II and is thought to be an important determinant of blood pressure and electrolyte homeostasis.27,28 Recent genetic linkage studies suggest that angiotensinogen is one of the candidate genes for hypertension.1–4 Indeed, recent findings suggest the pivotal role of angiotensinogen in high blood pressure regulation.7–9 Therefore, it is becoming increasingly important to study the molecular mechanism(s) involved in the regulation of the angiotensinogen gene. As previously

**Figure 5.** A, Typical example of the expression of brain angiotensinogen mRNA in SHRs (at the maintenance stage) transfected with AGE 2 or mismatched decoy ODNs. Brain tissue was harvested 72 hours after transfection. Lane 1, SHR brain transfected with mismatched AGE 2 decoy ODNs (20 μmol/L); lane 2, SHR brain transfected with AGE 2 decoy ODNs (20 μmol/L) (20 μg mRNA per each lane). B, Levels of mRNA expression were quantified and compared by densitometric analysis. AGE indicates AGE 2 decoy ODN–transfected SHRs (n=5); mis, mismatch decoy ODN–transfected SHRs (n=5). *P < 0.05 vs mismatched ODNs.

**Figure 6.** A, Brain concentration of angiotensinogen. B, Brain concentration of angiotensin II. Both concentrations were measured 2 days after transfection of AGE 2 decoy ODNs (n=8) or mismatched decoy ODNs (n=8). *P < 0.05 vs mismatched ODNs.

**Figure 7.** A, Competition assay of AGE 3 decoy ODNs by gel mobility shift assay for AGE 3 binding site. N indicates 32P-labeled AGE 3 decoy ODNs without nuclear extract; C, nuclear extract (50 μg) from SHR brain incubated with 32P-labeled double-stranded AGE 3 decoy ODNs for 30 minutes at room temperature without any competitor; and AGE 3, C plus AGE 3 decoy ODNs (2× or 4× excess). B, Gel mobility shift assay for AGE 3 binding site. N indicates 32P-labeled AGE 3 decoy ODNs without nuclear extract; SHR #1 through #3, nuclear extracts (50 μg) from SHR brain incubated with 32P-labeled double-stranded AGE 3 decoy ODNs for 30 minutes at room temperature; and WKY #1 through #3, nuclear extracts (50 μg) from WKY brain incubated as above. C, Binding activity of AGE 3 by densitometric analysis of gel mobility shift assay (SHRs, n=8; WKYs, n=8). *P < 0.01 vs WKYs.
described, the 5'-flanking region of the human angiotensinogen gene is important for tissue- and cell type–specific expression of the gene in vivo as well as in vitro. In human hepatocytes in vitro, cell type–specific activation of angiotensinogen gene transcription results from the cooperative interaction of a proximal promoter element (AGE 2; from −96 to −52) with AGE 3 (from −6 to +22), which is located around the transcriptional start site in the core promoter region. However, little is known about the molecular mechanism(s) of angiotensinogen gene regulation in vivo. Therefore, using a decoy strategy, we examined this issue and found that the transcriptional cis element AGE 2, rather than AGE 3, plays an important role in the control of hepatic angiotensinogen gene expression using a decoy strategy.

Currently, the decoy strategy is considered a useful tool in a new class of anti-gene strategies. Transfection of double-stranded ODNs corresponding to the cis sequence will result in the attenuation of the authentic cis-trans interaction, leading to the removal of the trans factors from the endogenous cis element, with subsequent modulation of gene expression (Figure 1). Therefore, the decoy approach enables investigation of gene regulation in vivo as well as in vitro by modulation of endogenous transcriptional regulation. Previously, we proved that the HVJ-liposome method prolonged the half-life of ODNs and concentrated them in the nuclei of cultured neuronal cells in vitro and in rat brains in vivo. We also confirmed the safety of this method by histological examination and behavioral observation. In this kinetic study, intracerebroventricular administration of FITC-labeled ODNs with HVJ-liposome produced a wider distribution of ODNs in the central nervous system in vivo than did administration of ODNs alone. This important experiment showed the broad distribution of FITC-labeled ODNs in the neuronal cells of the hypothalamic nuclei, subfornical organ, and some other periventricular regions, which are considered important areas in blood pressure regulation and which may be responsible for the decrease in blood pressure via angiotensinogen gene suppression.

Recent progress in molecular biology clearly indicates the existence of the components of a renin-angiotensin system in several tissues, including brain. Angiotensinogen, renin, angiotensin-converting enzyme (ACE), and the Ang II receptor have all been detected in brain. Furthermore, the intracerebroventricular infusion of angiotensin-converting enzyme inhibitors lowers the high blood pressure of SHR. In addition, the neurons in the anteroventral third ventricular regions are rich in immunoreactive Ang II. This raises the possibility that the brain is involved in blood pressure control by the regulation of angiotensinogen production. Thus, it is noteworthy to clarify the detailed molecular mechanisms of angiotensinogen production in the brain of a genetically hypertensive model animal.

AGE is the proximal promoter element and a binding site for AGF. This transcriptional factor was detected by Tamura et al in the HepG2 cell line and by us in rat liver. The present results are the first observations to detect this transcriptional factor in the rat brain tissue. To our knowledge, no report has been published that AGE 2 binding factor in the brain regulates other genes in addition to angiotensinogen. However, this transcriptional factor may induce activation of some other genes. Further experiments using molecular biological techniques are required to clarify this possibility and unknown gene activation.

The palindromic sequences of AGE 2 are well conserved between the rat and mouse angiotensinogen gene, and a previous study has shown that the palindromic sequence of the rat angiotensinogen gene is important for the formation of a specific complex with HepG2 nuclear extract. Overall, our results demonstrate that transfection of decoy ODNs against AGE 2, but not against AGE 3, of the angiotensinogen gene results in a transient decrease in the high blood pressure of SHRs, which suggests that the transcriptional cis element AGE 2, rather than the AGE 3 binding site, plays an important role in high blood pressure through the control of brain angiotensinogen production in SHRs.
This study also revealed the utility of gene transfer and decay technology for hypertension research, especially for evaluation of the tissue-specific functions of transcriptional factors of target gene regulation.

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