Regulation of Angiotensin II Receptors in Rat Brain During Dietary Sodium Changes

Kathryn Sandberg, Hong Ji, Kevin J. Catt

Abstract
Activation of the renin-angiotensin system by sodium deficiency is associated with reciprocal changes in the expression of angiotensin II receptors in adrenal glomerulosa and vascular smooth muscle cells. The effects of dietary sodium changes on the expression of brain angiotensin receptor subtype 1 (AT1) mRNAs were examined in rats maintained on normal, low, and high sodium intake for 3 weeks. Plasma aldosterone and renin activity were elevated in rats maintained on a low salt diet compared with normal rats and were reduced in rats maintained on a high salt diet. These results are consistent with previous findings on the effects of altered dietary sodium on the renin-angiotensin system. The expression of AT1A and AT1B receptor subtype mRNAs was determined by quantitative reverse transcriptase-polymerase chain reaction during changes in sodium intake. The results revealed that sodium deprivation enhanced the expression of AT1B receptors in decorticated brains by 164% compared with high sodium intake. Conversely, high sodium diet increased the expression of AT1A receptors by 155% in the brain compared with low sodium intake. These data suggest that AT1A and AT1B receptors play reciprocal roles in central mechanisms for the control of fluid homeostasis. Further analysis of the molecular biology of angiotensin II receptor regulation in the brain may provide new insights into the interplay between the renin-angiotensin system and blood pressure regulation and also into the role of angiotensin II in the pathogenesis of essential hypertension.

Key Words • angiotensin • receptors, angiotensin • diet, sodium-restricted • brain

The renin-angiotensin II system (RAS) plays a significant role in the neuroendocrine control of cardiovascular function.1 The octapeptide hormone angiotensin II (Ang II) is one of the major regulators of electrolyte balance and cardiovascular function and is one of the most potent circulating vasoconstrictor hormones. Ang II modulates peripheral vascular tone, enhances salt retention (via aldosterone release), and exerts central pressor and dipsogenic effects. It also has been postulated that abnormalities in the RAS could contribute to congestive heart failure, peripheral vascular disease, and essential hypertension.2,3

The cardiovascular neuronal centers within the brain are considered to mediate Ang II actions, including pressor, tachycardiac, and dipsogenic responses.4,5 Several lines of evidence indicate that the RAS operates in the brain as a long-term regulator of arterial pressure and sodium balance and is involved in the pathogenesis of hypertension. In the renal hypertensive dog, elevated levels of Ang II were found in the cerebrospinal fluid and were not altered by nephrectomy.6 Ang II antagonists or angiotensin converting enzyme inhibitors normalized blood pressure in hypertensive rats,7 and lesions in brain regions possessing Ang II receptors attenuated experimental hypertension.8 Furthermore, Ang II receptors were found to be associated with various cardiovascular regulatory sites in the brain.9,10 These receptor sites are mainly located in the circumventricular organs, where they can detect blood and/or cerebrospinal fluid levels of Ang II.

Recently, two distinct Ang II receptor genes have been cloned in the rat.11,12 These two receptor subtypes share a high degree (95%) of amino acid homology. However, subtype 1B (AT1B) receptors consistently express threefold to fivefold greater affinity for Ang II agonists and antagonists than subtype 1A (AT1A), and dose-related differences between the two receptor subtypes are present during Ang II-induced calcium signaling.12 Furthermore, AT1A and AT1B receptors have been shown to be differentially regulated in the pituitary gland.13 The primary sites of pressor action for Ang II in the rat brain appear to be in the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT).1,5 Therefore, it is of interest that AT1B receptors recently have been shown to be more abundant than AT1A in the SFO and OVLT.14

Dietary sodium restriction is known to activate the RAS and to increase circulating levels of Ang II.15,16 Experimental evidence suggests that an elevated level of Ang II in the brain will increase the expression of angiotensin receptors and is associated with an increase in blood pressure and pressor responses, the induction of thirst, and altered resistance of the renal and mesenteric vascular beds.17,18 In this study, we examine the effects of dietary sodium changes on the expression of both AT1 subtype mRNAs in the decorticated rat brain to address the molecular mechanisms involved in Ang II receptor regulation in the brain.

Methods

Materials

[^32P]Deoxyctydine 5'-triphosphate (dCTP) (>6000 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Oligonucleotide primers were synthesized by Lofstrand, Gaithersburg, Md., or Midland, Midland, Tex. Superscript RNase H- reverse transcriptase and transcription buffer were
purchased from GIBCO/BRL, Gaithersburg, Md. Taq polymerase was obtained from Perkin-Elmer Cetus, Norwalk, Conn. RNase-free DNase was obtained from Stratagene, La Jolla, Calif. β-Actin was purchased from Clontech, San Diego, Calif. Three groups each of 33 male 50-day-old Sprague-Dawley rats (Charles River, Inc, Wilmington, Mass) were maintained on low sodium (0.03% to 0.05%), normal (0.2%), and high sodium (3.15% to 3.45%) diets (Purina Mills, St Louis, Mo) for 3 weeks. All animal procedures were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

**Hormone Assays**

Rats were killed by decapitation, and approximately 5 mL of blood was collected in the presence of EDTA (5 mmol/L) for determination of aldosterone, renin, and corticosterone levels. Plasma aldosterone concentrations were measured by radioimmunoassay after extraction with methylene chloride and fractionation by LH-20 chromatography.19 Renin activity was assayed as previously described. Plasma corticosterone concentrations were also measured by radioimmunoassay according to published methods.21

**RNA Preparation**

After decapitation, the brain was decorticated and immediately frozen in liquid nitrogen. Total RNA was prepared by the method of Chomczynski and Sacchi22 from six pools of five to six brains each for control, low, and high salt groups. Genomic DNA contamination was removed from total RNA samples (10 μg) by a 10-minute incubation with RNase-free DNase (10 U) at 37°C in transcription buffer, followed by phenol extraction. Total RNA (20 μg per lane) was electrophoresed through a denaturing 2.2 mol/L formaldehyde/1% agarose gel and stained with ethidium bromide for assessment of RNA quality.

**cDNA Synthesis**

Total RNA (5 μg) was reverse transcribed with oligo(dT) primers and reverse transcriptase in a 20-μL reaction volume for 60 minutes at 42°C, as previously described.12

**Synthesis of Polymerase Chain Reaction Products**

Rat brain cDNA was primed with oligonucleotides (10 pmol each) based on the sequences of β-actin and AT1A and AT1B receptors (Table 2) and was amplified by polymerase chain reaction (PCR) for 20 (β-actin) or 27 (AT1A and AT1B) cycles unless otherwise specified. The amplification conditions were as follows: denaturation at 94°C for 45 seconds; primer annealing at 62°C, 53°C, or 57°C (β-actin, AT1A, and AT1B, respectively) for 45 seconds; and extension at 72°C for 1 minute using Taq polymerase in the presence of MgCl2 (concentration specified in Table 2) in a Perkin-Elmer Cetus DNA thermal cycler.

**cDNA Probes**

cDNA probes were prepared by PCR19 in the presence of 1 ng substrate (cloned β-actin, AT1A, or AT1B; [α-32P]dCTP (100 μCi per reaction), 2 μmol/L each of deoxyadenosine 5'-triphosphate, thymidine 5'-triphosphate, and deoxyguanosine 5'-diphosphate; 1.5 mmol/L (β-actin) or 4 mmol/L (AT1A and AT1B) MgCl2; 10 pmol each of antisense and sense primers (Table 2); and 2.5 U of Taq polymerase in a total volume of 20 μL. After PCR, the labeled DNA was separated from free nucleotides on nucleotide-push columns (Stratagene).

**Quantitation of Angiotensin Receptor Expression**

Ten microliters of the 50-μL reaction mixture was electrophoresed through 1% agarose gel and blotted onto Nytran membranes with a pressure blower (Stratagene). The blot was probed with labeled cDNA representing the open reading frame of AT1A or AT1B for Southern analysis.12 After hybridization and washing at high stringency, washed membranes were scanned using a radioanalytic imaging system (AMBIS Systems, Inc, San Diego, Calif).12

**Results**

The recent demonstration of the predominance of AT1B receptors in the circumventricular regions of the rat brain14 led us to examine whether angiotensin receptors in the brain were differentially regulated by alterations in sodium diet. Male Sprague-Dawley rats were maintained on low, normal, or high sodium diets for 3 weeks before death. To confirm the effectiveness of dietary sodium restriction, we measured plasma levels of renin activity, aldosterone, and corticosterone (Table 1). Sodium deprivation markedly increased aldosterone production (18-fold) and caused a significant increase in renin activity (1.4-fold). On the other hand, sodium loading substantially reduced aldosterone (>75%) and renin activity (>73%) compared with the group maintained on a normal diet. Rats maintained on the high sodium diet had increased levels (1.6-fold) of corticosterone compared with the control group. These results are consistent with previous reports demonstrating the effects of dietary sodium on the RAS.24,26

Immediately after death, rat brains were removed, decorticated (because the cortex is known to possess few Ang II receptors24), and frozen in liquid nitrogen. Total RNA was isolated from individual rat brains and subjected to electrophoresis to ascertain RNA integrity before cDNA was synthesized. No detectable degradation was observed in samples used for cDNA synthesis and amplification by the PCR. Oligonucleotide primers (Table 2) were chosen from the 3' and 5' untranslated regions (areas of these receptors that share <65% homology) to selectively amplify the coding region of AT1A or AT1B receptor cDNA. In initial experiments, annealing temperature and MgCl2 concentration were optimized for each set of primers to determine maximal primer efficiency (data not shown). Control experiments revealed that AT1A-specific primers did not amplify purified AT1B cDNA, nor did AT1B-specific primers amplify purified AT1A cDNA (<50 cpm/μg RNA). Genomic DNA contamination of our RNA preparations was minimized by treatment of RNA with RNase-free DNase before cDNA synthesis. Furthermore, AT1A- and AT1B-specific primers did not detectably

**Table 1. Effect of Dietary Sodium on Concentrations of Plasma Aldosterone, Renin Activity, and Corticosterone Levels**

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Aldosterone, ng/dL</th>
<th>Renin Activity, ng Ang I/mL/h</th>
<th>Corticosterone, μg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low sodium</td>
<td>530±37</td>
<td>4.9±0.65</td>
<td>16±3.7</td>
</tr>
<tr>
<td>Normal sodium</td>
<td>29±3.6</td>
<td>3.6±0.80*</td>
<td>15±2.6</td>
</tr>
<tr>
<td>High sodium</td>
<td>6.8±0.94</td>
<td>0.96±0.14</td>
<td>24±4.7</td>
</tr>
</tbody>
</table>

*Mean renin activity of control group is skewed because of two rats with unusually high renin levels; median value for normal sodium control group is 2.9 (ng Ang I/mL/h).
amplify RNA that had not been reverse transcribed (<80 cpm/μg RNA).

The amplified products were analyzed by Southern blotting using specific angiotensin receptor probes that were synthesized by PCR in the presence of [α-32P]dCTP (Table 2). An equivalent amount of probe was used to directly compare the hybridization signal obtained with AT1A- and AT1B-specific probes. After washing under identical conditions, the hybridization signal was quantitated by using a radioanalytic imaging system. We used a quantitative PCR assay to examine the effects of salt diet on angiotensin receptor mRNA because expression levels of angiotensin mRNA are relatively low in the brain, and PCR has the distinct advantage of being several-fold more sensitive than Northern analysis.

In Fig 1, the amount of radioactivity of the 1100-bp fragments amplified by PCR is shown to be linearly related to amplification cycle number for both AT1A and AT1B receptors. Under these conditions, incorporation was measurable at cycle 10 and was linear through cycle 30. Fig 2 demonstrates that there is also a linear relation between the amount of cDNA and radioactivity in the amplified fragments for both receptors after 27 cycles. Thus, product accumulation is dependent on the amount of substrate under these assay conditions between 15 and 500 ng RNA.

To directly compare the AT1A with AT1B receptor expression, we examined product accumulation from 500 ng of reverse-transcribed RNA after 27 cycles. Fig 3 demonstrates that rat AT1B receptor expression is increased after 3 weeks of sodium deprivation compared with control (142%, \( P<.05 \)) and high sodium (164%, \( P<.05 \)) diets. On the other hand, AT1A receptor expression was conversely affected by the altered sodium diets; high salt intake increased AT1A receptor expression compared with control (135%, \( P<.05 \)) and low salt (155%, \( P<.05 \)) diets.

**Discussion**

Previous work has shown that the Ang II–induced drinking response is decreased in estrogen-treated rats and that this change is related to a reduction in the number of Ang II receptors in the brain. The latter finding, together with the observations that estrogen selectively attenuates AT1B receptor expression in the pituitary and is predominantly expressed in brain regions involved in thirst, suggests that AT1B receptors

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**TABLE 2. Primer Sequences for Amplification of Angiotensin cDNA During Polymerase Chain Reaction and Probe Sequences for Detection of Amplified DNA on Southern Blots**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Sense and Antisense Primers</th>
<th>Base Pairs</th>
<th>Bases Spanned</th>
<th>([\text{MgCl}_2], \text{mmol/L})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1A</td>
<td>S: TGAGACCAACTCAACCCAGA</td>
<td>244-263</td>
<td>1137</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>AS: GCATTACATTGCCAGTTG</td>
<td>1363-1381</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1B</td>
<td>S: CACCTCGCCAAGGAGAC</td>
<td>132-149</td>
<td>1119</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>AS: CACTTGCAAGCTTTGACCC</td>
<td>1233-1251</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>S: GAGAAGATGACACCCAGATCATGT</td>
<td>1702-1721</td>
<td>401</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>AS: ACTCCATGCCAGGAAAGGAAG</td>
<td>2569-2588</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AT1A indicates angiotensin receptor subtype 1A; S, sense; AS, antisense; and AT1B, angiotensin receptor subtype 1B.

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**Figure 1.** Semilogarithmic plot shows product accumulation (counts per minute [cpm]×10^{-3}/μg RNA) vs cycle number. cDNA synthesized from 500 ng of angiotensin receptor subtype 1A (A) or 1B (○) RNA was selectively amplified with subtype-specific primers (Table 2) as a function of amplification cycle. Results are mean±SEM; n=4.
AT, B were 4250, 409, and 311 cpm/mg RNA, respectively.

Analytic imaging system. Data are expressed as amount of AT W

were hybridized with radiolabeled 0-actin, AT 1A-, and AT 1B-
specific primers (Table 2). Amplified products were electropho-

amplified for 27 cycles by PCR using 0-actin, AT 1A-, and AT 1B-
specific probes. Hybridization signals were analyzed by a radio-

observations. Control levels of production for 0-actin, AT, and

mulation (cpm//ig RNA); each point is the mean±SEM of six

reseed and transferred to Nytran membranes. Southern blots

per sample) was

cDNA synthesized from total RNA (5 µg per sample) were selectively amplified for 27 cycles. Results are mean±SEM; n=4.

are involved in the mediation of drinking behavior. It

remains to be seen whether this reciprocal regulation of

angiotensin receptors is found in all brain regions

possessing Ang II receptors or is limited to specific

structures.

In studies that did not distinguish between angioten-
sin subtypes, sodium deprivation was shown to increase

the expression of angiotensin mRNA in the adrenal,11

and bilateral nephrectomy markedly reduced the ex-

pression of angiotensin receptor mRNA in the adrenal

and brain stem.29 Thus, it will be interesting to deter-

mine whether AT Ta and AT IB receptors are reciprocally regulated under other conditions that alter the RAS in

the brain and other tissues such as the adrenal, as well

as in other species.30

Our findings also raise the consideration that recip-

rocal regulation of individual subtypes could account

for the lack of effect of altered sodium intake on total

angiotensin receptor expression. For example, it was

reported that rats maintained on a low sodium diet for

4 weeks had no differences in the level of angiotensin

receptor expression in the kidney, liver, and brain.31

Clearly, it will be important to evaluate angiotensin

receptor subtype expression in the brain, aorta, and

kidney under conditions that alter the RAS, such as

prolonged infusion of Ang II and treatment with DuP

753 or captopril, and that are not associated with a

change in total angiotensin receptor expression.

Variations in sodium balance are known to influence

angiotensin receptor expression in opposite ways de-

pending on the tissue source. In vascular tissues, which

possess primarily AT 1A receptors,13 pressor responsiveness

to Ang II and Ang II receptor expression are

attenuated during sodium restriction. The opposite ef-

fects of sodium diet are observed in the adrenal glom-

erula, which possesses predominantly AT 1B recep-

tors.13 The aldosterone response and Ang II receptor

expression are potentiated during sodium restriction

and decreased by sodium loading.31 The selective regu-

lation of angiotensin subtype mRNA expression pro-

vides a mechanism that could account for the dual

actions of Ang II on aldosterone secretion and vasocon-

striction and could minimize the vascular effects of

altered sodium during variations in renin secretion

associated with the control of sodium balance.

It is of note that although AT 1A and AT 1B receptors

share 95% amino acid homology, potentially important

differences do exist between their protein structures.

Most of the structural differences are found in the third

cytoplasmic loop (an area involved in guanine nucleo-
tide–dependent protein interaction) and the carboxy

terminal intracellular domain, and include the presence

of two additional potential protein kinase C phosphor-

ylation sites in AT 1B compared with AT 1A. Thus, func-

tional differences between these receptors could lie at

the amino acid level. Alternatively, differential regula-

tion could be determined by the genomic structure of

the receptor. Although the structure of the AT 1A recep-

tor gene has recently been reported,32 the genomic

structure of the AT 1B receptor has not been determined.

It will be important to study the differential regula-

tion of these two distinct genes under conditions that alter

the activity of the RAS. Such studies could lead to

further insights into the relevance of these genes in

hypertensive disease states and possibly to new and

more specific therapies, as well as furthering our under-

standing of central neuronal and neuroendocrine path-

ways that control the regulation of sodium balance,

vascular reactivity, and peripheral sympathetic drive.

In conclusion, the present studies have demonstrated

that changes in angiotensin receptor expression in the

brain occur during altered sodium balance and are

characterized by reciprocal changes in mRNA levels for

the AT 1B and AT 1A receptors. Whereas sodium restric-

tion led to increases in AT 1B receptor expression, alter-
ations in dietary sodium caused converse changes in AT_{1A} receptor expression. It is likely that such reciprocal regulation of brain angiotensin receptors contributes to the maintenance of fluid homeostasis during changes in sodium intake. An understanding of the individual roles of the Ang II receptor subtypes in the physiology and pathophysiology of the RAS may provide new information regarding the regulation of blood pressure and pathogenesis of arterial hypertension.

Acknowledgments

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