Quantitation of Hypothalamic Atrial Natriuretic Peptide Messenger RNA in Hypertensive Rats

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Previous studies from our laboratory have shown that spontaneously hypertensive rats have increased atrial natriuretic peptide stores and reduced norepinephrine release from nerve terminals in the anterior hypothalamus. We have postulated that atrial natriuretic peptide inhibits norepinephrine release in anterior hypothalamus, reducing excitation of sympathoinhibitory neurons, increasing sympathetic outflow, and elevating blood pressure in this model. The current study tested the hypothesis that atrial natriuretic peptide messenger RNA (mRNA) transcript levels are increased in anterior hypothalamus of spontaneously hypertensive rats compared with normotensive Wistar-Kyoto rats. Atrial natriuretic peptide mRNA in hypothalamic regions was measured by the quantitative polymerase chain reaction technique using a p-SELECT mutant atrial natriuretic peptide RNA as an internal standard. Atrial natriuretic peptide mRNA from hypothalamic regions of spontaneously hypertensive and Wistar-Kyoto rats and the internal standard were coamplified in a single reaction in which the same primers were used. Since the polymerase chain reaction product of the internal standard contained a new EcoRI restriction site, it could be distinguished from the atrial natriuretic peptide mRNA product by EcoRI digestion after the polymerase chain reaction. We found regional inhomogeneity of atrial natriuretic peptide mRNA in the hypothalamus of spontaneously hypertensive and Wistar-Kyoto rats, but we found no significant differences in atrial natriuretic peptide mRNA levels in anterior, posterior, or ventral hypothalamic areas between spontaneously hypertensive rats and Wistar-Kyoto rats fed normal (1%) or high (8%) NaCl diets. These data do not support the hypothesis that increased atrial natriuretic peptide stores are related to increased gene transcription. (Hypertension 1992;19:296–300)

KEY WORDS • polymerase chain reaction • atrial natriuretic peptides • sodium-dependent hypertension • messenger RNA • spontaneously hypertensive rats

Atrial natriuretic peptide (ANP), the natriuretic and vasorelaxant peptide hormone originally described in mammalian atria,1,2 is also found in many other tissues, including ventricle,3 pituitary, lung,4 and brain.5-12 The genes for human,13-15 mouse,15 and rat16 ANP have been cloned and their nucleotide sequences determined. Each ANP gene consists of three coding blocks (exon 1, 2, and 3) separated by two intervening sequences (intron 1 and 2) from which the structure of prepro-ANP has been deduced.

Immunoreactive ANP has been detected in various brain regions by radioimmunoassay of extracted tissue and by immunocytochemistry.5,7,9 ANP messenger RNA (mRNA) and ANP receptors have also been described, indicating that an intact ANP synthetic, processing, and effector system is present in rat brain.5-12 Immunocytochemical data show that ANP-containing cell bodies are present in highest density in the anterior hypothalamic region adjacent to the anteroventral tip of the third ventricle.9,17 This region is involved in the development and maintenance of experimental hypertension and in fluid and electrolyte balance.9,17

Previous studies from our laboratory have shown that NaCl-sensitive spontaneously hypertensive rats of the Okamoto strain (SHR-S) have increased ANP stores12 and reduced norepinephrine release from nerve terminals in the anterior hypothalamic area (AHA).18 We have postulated that ANP inhibits norepinephrine release in AHA, reducing excitation of sympathoinhibitory neurons, increasing sympathetic outflow, and elevating blood pressure.19 The current study tested the hypothesis that ANP mRNA expression is increased in AHA of SHR-S compared with normotensive Wistar-Kyoto (WKY) rats. To quantify the low levels of ANP mRNA in hypothalamic tissue, we used an ultrasensitive quantitative polymerase chain reaction (PCR) technique that enables us to detect subnanogram amounts of ANP mRNA in brain. We demonstrated a regional inhomogeneity of ANP mRNA in the hypothalamus of SHR-S and WKY rats, but there were no significant differences in ANP mRNA levels in AHA and posterior (PHA) and ventral (VHA) hypothalamic areas between SHR-S and WKY rats fed normal (1%) or high (8%) NaCl diets.

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Methods

Animal and Tissue Preparation

Male SHR-S and WKY control rats were obtained from IBU-3 colony, Taconic Farms, Germantown, N.Y., at 7 weeks of age. Three days after arrival, half of the rats in each group were placed on an 8% NaCl diet (Purina Chow, ICN Biochemicals, Costa Mesa, Calif.); the other half remained on the basal 1% NaCl diet (diet 5001,Ralston Purina, St. Louis, Mo.). All rats were maintained four per cage at constant humidity (60±5%), temperature (24±1°C), and 12-hour light/dark cycle. Food and water were available ad libitum throughout the study. Systolic blood pressure was measured weekly by the tail-cuff method with a blood pressure analyzer and physiograph recorder (IITC, Inc., Woodland Hills, Calif.). Three weeks after initiation of the special diets, rats were killed by decapitation without anesthesia. Brains were removed immediately, and the hypothalamus was dissected into VHA (7.5-9 mg), AHA (10-11 mg), and PHA (9-10 mg) as previously described.18 Total tissue RNA was extracted by the guanidine thiocyanate method described by Chomczynski and Sacchi.20 The precipitated RNA was dissolved in H2O, quantitated by absorbance at 260 nm, and stored at −80°C until assay. Each hypothalamic region from each experimental animal was processed separately.

Generation of Mutant p-SELECT Atrial Natriuretic Peptide DNA and RNA

p-SELECT mutant ANP DNA with an EcoRI restriction site (Figure 1A) was generated using the Promega Altered Site in vitro Mutagenesis System (Promega Corporation, Madison, Wis.). Briefly, a 0.7 kb fragment of ANP complementary DNA (cDNA) (kindly supplied by Dr. R. Weigand, Monsanto Corporate Research, St. Louis, Mo.) was first inserted into the phagemid vector p-SELECT-I. A 20mer ANP mutagenic oligonucleotide 5'-ACTGGGGAATTCAACCCGTC-3' (Oligos Etc. Inc., Guilford, Conn.) was used to generate a single site mutation following the procedures described by the manufacturer. Using the p-SELECT mutant ANP DNA plasmid as the template, p-SELECT mutant ANP RNA was then generated by the in vitro transcription method (Promega). The p-SELECT mutant ANP RNA was stored in 720 ng/10 µl aliquots at −80°C until use. The p-SELECT mutant ANP RNA was used as the internal standard in reverse transcription (RT) and quantitative PCR (Figure 1B).

Reverse Transcription and Quantitative Polymerase Chain Reaction

RT of ANP mRNA to ANP cDNA and subsequent PCR amplification were performed in the same reaction tube using the GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, Conn.). Before RT, serial dilutions of mutant p-SELECT mutant ANP RNA (72, 7.2, 0.72, and 0.072 ng/µl) were first mixed with a fixed amount (1.5 µg/µl) of tissue RNA from AHA, PHA, or VHA. A synthesized downstream 33mer oligonucleotide (5'-CCGACGTGTCAGGCGATGCTTCTATTCGTC-3'), which spanned the junction of exon 2 and 3 of ANP genomic DNA, was used as the primer in the RT for the first strand cDNA synthesis; the same downstream 33mer primer and a 28mer upstream primer (5'-CTGATGGATTCAAGAAGCCTGTCTA- GACC-3'), which spanned the junction of exon 1 and 2 of ANP genomic DNA, were used in the PCR for the ANP cDNA amplification. During RT and PCR, [32P]deoxyribocytosinetriphosphate (dCTP) was used as one of the substrates to label the PCR products. Because of the optimal design of the primers for the RT and PCR, only the ANP mRNA and cDNA could be used as templates during the RT and PCR. RT and PCR gave a 357-bp product when authentic ANP mRNA and p-SELECT mutant ANP RNA were amplified. PCR products were extracted with phenol/chloroform and precipitated by ethanol. The precipitated DNA was reacted with EcoRI for 1 hour and then loaded on 2.5% agarose gel for electrophoresis. The RT and PCR product of p-SELECT mutant ANP RNA was cut by EcoRI to form 209- and 148-bp fragments, but the product of tissue ANP mRNA remained intact.
throughout the enzymatic reaction. The gels were stained with ethidium, and the 357-, 209-, and 148-bp bands were cut out from the gels and counted for $[^{32}P]$ radioactivity. The quantity of radioactivity in the 209- and 148-bp bands was plotted against the amount of p-SELECT mutant ANP RNA. The amount of tissue ANP mRNA was then quantitated by extrapolating against the standard curve generated with the p-SELECT mutant ANP RNA.

**Statistical Analysis**

Results were expressed as mean±SEM. Data were analyzed using the CRUNCH statistical package on an IBM PC/AT computer. Statistical comparisons of ANP mRNA levels were made using analysis of variance (ANOVA). Differences were reported as significant if the value of $p<0.05$.

**Results**

Four SHR-S and four WKY rats fed either 1% or 8% NaCl diets were studied. In SHR-S, the high NaCl diet caused a significant increase in systolic blood pressure (196±4 mm Hg) compared with SHR-S fed the basal NaCl diet (173±5 mm Hg; $p<0.05$). The high NaCl diet had no significant effect on systolic blood pressure in WKY rats (125±4 versus 129±4 mm Hg; 1% versus 8% NaCl, respectively).

A schematic outline of the in vitro mutagenesis and quantitative PCR method are given in Figure 1. The single base mutation site of p-SELECT mutant ANP DNA was confirmed by nucleotide sequence analysis (not shown). Figure 2 shows a representative ethidium-stained 2.5% agarose gel of quantitative PCR with AHA samples. Digestion with EcoRI selectively cut DNA fragments derived from the p-SELECT mutant ANP RNA into two subfragments of 148 and 209 bp in length (Figure 2A). An individual linear regression curve ($r>0.95$) (used as standard curve) was generated for the calculation of ANP mRNA levels in each sample after the log-log transformation of $[^{32}P]$ radioactivity and internal standard p-SELECT mutant ANP RNA (Figure 2B). For measurement of the ANP mRNA content in each tissue sample, four aliquots of a fixed amount of tissue RNA (1.5 μg/μl) from the same sample were mixed with four dilutions of p-SELECT mutant ANP RNA (72, 7.2, 0.72, and 0.072 ng/μl) before RT and PCR. After gel electrophoresis, the $[^{32}P]$ radioactivity of the four 357-bp bands from the same tissue sample were counted and averaged. The absolute amount of tissue ANP mRNA was calculated from the averaged $[^{32}P]$ radioactivity of the 357-bp bands versus the standard curve generated from the p-SELECT mutant ANP RNA.

The levels of ANP mRNA in PHA measured by quantitative PCR were significantly lower than in AHA and VHA in all animals studied ($p<0.05$, ANOVA). The levels of ANP mRNA in VHA were slightly but not significantly greater than in AHA. There were no significant differences in AHA, PHA, or VHA ANP mRNA levels between SHR-S and WKY rats fed normal or high NaCl diets for 3 weeks (Figure 3).

**Discussion**

Quantitative PCR has recently been used to accurately determine the amounts of low abundance specific mRNAs in small (less than 0.1 ng) tissue samples. In the current study, we generated a p-SELECT mutant ANP RNA for use as the internal standard for the quantitation of low levels of ANP mRNA in brain tissue using quantitative PCR. The principle of this method is based on the coamplification of an in vitro–generated p-SELECT mutant ANP RNA differing by a single base exchange from authentic tissue ANP mRNA. The p-SELECT mutant ANP RNA was added to samples of tissue ANP mRNA and was used as an internal standard during RT and PCR. After RT and PCR, the p-SELECT mutant ANP RNA produced a mutant ANP cDNA with a new EcoRI restriction site. Since a change in any of the variables in RT and PCR (e.g., concentrations of reverse transcriptase and DNA polymerase, deoxynucleotriphosphate, magnesium,
FIGURE 3. Bar graphs show anterior hypothalamic area (panel A), posterior hypothalamic area (panel B), and ventral hypothalamic area (panel C) atrial natriuretic peptide (ANP) messenger RNA (mRNA) levels of 1% or 8% NaCl-fed NaCl-sensitive spontaneously hypertensive rats (SHR-S) and Wistar-Kyoto (WKY) rats measured by quantitative polymerase chain reaction. Results are presented as mean±SEM. Units are ANP mRNA/tissue RNA (ng/µg).

and primers; annealing, extension, and denaturing temperatures; PCR cycle length and cycle number) affects the yield of quantitative p-SELECT mutant ANP RNA and authentic tissue ANP mRNA (or mutant or authentic ANP cDNA after RT) equally, relative ratios of the two were preserved with amplification. The p-SELECT mutant ANP DNA was distinguished from the authentic ANP cDNA by restriction enzyme (EcoRI) digestion after PCR (Figure 1D). The relative amount of authentic ANP cDNA versus p-SELECT mutant ANP cDNA was measured by direct scanning of ethidium-stained gels and by incorporation of [3P] radiolabeled dCTP. Because the starting concentration of the p-SELECT mutant ANP RNA was known, the initial concentration of the tissue ANP mRNA could be determined.

Several lines of evidence indicate that brain ANP is altered in SHR compared with normotensive control rats. Studies from a number of laboratories have demonstrated that the ANP content of the hypothalamus, pons, and septum is significantly elevated in SHR compared with age-matched WKY controls.24–27 Our previous studies demonstrated that ANP stores are significantly elevated in AHA, a brain region that modulates sympathetic outflow and gives rise to a depressor response when stimulated, in SHR-S on both normal and high NaCl diets compared with those in WKY rats.12 In contrast, there were no significant differences in ANP content of the PHA, VHA, pons, or medulla among the 1% or 8% NaCl-fed SHR-S and WKY rats. In the current study, using quantitative PCR, we demonstrated the presence of ANP mRNA in the hypothalamus of SHR-S and WKY rats. These data gave evidence that nuclei in the hypothalamus previously shown to store ANP very likely synthesize this peptide on locally produced ANP mRNA templates.

The finding of regional inhomogeneity of ANP mRNA content in subdivisions of hypothalamus suggests a regional inhomogeneity of ANP synthesis within the hypothalamus. Previous immunocytochemical studies of the distribution of ANP neurons in the hypothalamus support our current observations of a regional inhomogeneity of ANP synthesis (reflected by ANP mRNA content) within the hypothalamus. ANP immunoreactive cell bodies that are stained by antibodies to ANP are most abundant in the periventricular preoptic nucleus, paraventricular nucleus, and anterior hypothalamic area, which are included in the anterior hypothalamic dissection in the current study; the arcuate nucleus, which is included in the ventral hypothalamic dissection in the current study; and the median mamilary nucleus.58 The concurrence between regional hypothalamic ANP mRNA levels and distribution of ANP neurons identified by immunocytochemical techniques is greater than that between ANP mRNA levels and ANP stores quantitated by extraction of tissue because the former measures reflect cell bodies only and the latter, mainly nerve terminals.

In the present study, we did not find significant differences in AHA, PHA, or VHA ANP mRNA levels between SHR-S and WKY rats fed normal or high NaCl diets. ANP mRNA levels in AHA, PHA, and VHA were not correlated with the ANP stores in these hypothalamic regions of SHR-S and WKY rats.12 There are several possible explanations for the discrepancy between the ANP peptide content and ANP mRNA levels in subdivisions of hypothalamus. Since quantitative PCR measured only steady-state levels of ANP mRNA in tissue, the kinetics of ANP mRNA turnover (e.g., synthesis and degradation rates) were not elucidated by the current study. Second, the loci of prepro-ANP synthesis (cell bodies containing ANP mRNA) and storage of releasable active ANP peptide (nerve terminals) within the hypothalamus might differ. In addition, the regional control of ANP release from nerve terminals might differ in various subdivisions of hypothalamus. Future studies using another method or methods, such as nuclear run-off analysis, in situ hybridization, and immunocytochemistry, are needed to elucidate the relation between ANP gene expression and storage patterns and their regulation in various regions of hypothalamus.

References
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