Altered Structure, Regulation, and Function of the Gene Encoding the Atrial Natriuretic Peptide in the Stroke-Prone Spontaneously Hypertensive Rat

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Abstract—Through the genotype/phenotype cosegregation analysis of an F2 intercross, from the crossbreeding of stroke-prone spontaneously hypertensive rats (SHRSP) and stroke-resistant spontaneously hypertensive rats (SHR), we previously identified a quantitative trait locus for stroke on rat chromosome 5 (STR2) that colocalized with the genes encoding atrial and brain natriuretic peptides (ANP and BNP) and conferred a stroke-delaying effect. To further characterize ANP and BNP as candidates for stroke, we performed additional studies. Comparative sequence analysis revealed point mutations in both the coding and regulatory regions of ANP, whereas no interstrain differences were found for BNP. In in vitro studies in COS-7 and AtT-20 cells that were performed to test the relevance of a G→A substitution at position 1125, a Gly→Ser transposition in the SHRSP pro-ANP peptide resulted in different posttranslational processing of the SHRSP ANP gene product that was also associated with higher cGMP production (P<0.05). Furthermore, an analysis of a 5′ end mutation affecting a PEA2 regulatory binding site in the 5′ untranslated regulatory sequence of SHRSP ANP demonstrated a significantly lower ANP promoter activation in endothelial cells (P<0.05 versus the SHR ANP). In addition, the expression of ANP was significantly reduced in the brain, but not in the atria, of SHRSP compared with SHR (P<0.0001). No differences were detected with regard to BNP expression. The present results reveal substantial differences in ANP, but not BNP, structure and product among SHR and SHRSP, which supports a role of ANP in the pathogenesis of stroke in the SHRSP animal model. (Circ Res. 1999;85:900-905.)

Key Words: genetics, atrial natriuretic peptide, stroke, natriuretic peptide

Stroke remains an area of substantial, unmet medical need, in part because of the rather sketchy understanding we have of its cause and responsible pathogenic mechanisms. Although hypertension is recognized as the most significant risk factor for stroke, twin and family studies, as well as epidemiological surveys, have consistently provided evidence for an important genetic component to its cause.1-6

The genetic dissection of a complex trait like stroke has been recently brought into our reach through the development of new molecular genetic methods and techniques, combined with the use of highly inbred animal models for this phenotype. In this regard, the stroke-prone spontaneously hypertensive rat (SHRSP), obtained through selective breeding from the stroke-resistant spontaneously hypertensive rat (SHR), represents a suitable model for stroke.7 Interestingly, a number of characteristics associated with stroke in this model are also found in human stroke, such as high blood pressure and dietary factors (e.g., high Na+, low K+, low protein diets), all of which play a critical, permissive role in the development of stroke in rats8,9 and in humans.10 Furthermore, the histopathological aspects of the cerebrovascular lesions in SHRSP resemble those observed in a rare human form of inherited stroke, called CADASIL.11

To elucidate the genetic underpinnings of stroke, we recently performed a genotype/phenotype cosegregation analysis by using an F2 cohort obtained through the intercrossing of SHRSP and SHR strains. We used latency until the manifestation of stroke under a stroke-permissive dietary regimen (“Japanese-style diet”: low protein, low K+, high Na+) as the quantitative phenotype, and we found clear evidence for the existence of genes that specifically and independently of blood pressure levels contributed to the pathogenesis of stroke.12 In particular, we were able to detect 3 major stroke-relevant loci or quantitative trait loci on rat chromosomes 1, 4, and 5 that when combined accounted for about one third of the overall genetic variance.
The locus identified on rat chromosome 5 was found to confer a significant protective effect against stroke in the presence of the SHRSP allele. Its peak LOD score colocalized with the genes encoding atrial and brain natriuretic peptides (ANP and BNP, respectively). These peptides have important cardiovascular functions (they induce vasorelaxation, diuresis, and natriuresis) and may therefore be viewed as legitimate candidate genes for cardiovascular diseases, including stroke. In addition, both ANP and BNP are expressed in cardiac and cerebral tissues, although to different degrees. ANP is mainly produced by the atria, whereas BNP is secreted primarily by the ventricle. Although BNP is widely distributed in the brain, ANP is mainly localized in hypothalamic and brain stem areas, which are involved in body fluid volume and blood pressure regulation.

The aim of the present study was to perform a detailed comparative analysis of ANP and BNP in our SHR and SHRSP strains, with particular regard to structure, regulation, and function, in an attempt to further support the candidacy of these genes as representing the substrate of the previously characterized locus STR2.

Materials and Methods

The SHR and SHRSP strains used for the present study have been described previously.

Sequence Analysis

The complete ANP and BNP coding sequences of SHR and SHRSP and a region of 641 bp upstream from the CAP site for ANP were amplified through the use of primers generated on the basis of the published sequence information. Direct sequencing was performed with an ABI377 sequencing apparatus (Perkin–Elmer Cetus).

Genotyping Assay and Single-Strand Conformational Polymorphism

A single–base pair mutation, found at position 1125 of ANP in SHRSP, was analyzed with the use of a BstUI polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) assay. Another base pair mutation, detected at position −463 (within the 5′ untranslated regulatory sequence [URS] of ANP) in SHRSP, was analyzed with the use of single-strand conformational polymorphism (SSCP).

ANP cDNA Subcloning and Transfection Studies: Western and Northern Blotting Analyses of Transfected Cells

The full-length SHR and SHRSP ANP cDNAs were amplified from reverse transcribed mRNA, inserted into the PCR TM3 vector (Invitrogen), and used to transfet COS-7 and AtT-20 cells. At 2 days after transfection, cellular extracts were obtained for protein analysis, and total RNA was extracted for Northern blotting.

Analysis of ANP on Tissues: Reverse Transcription–PCR and Northern and Western Blotting

Total RNA was used to obtain the ANP cDNA and for Northern blotting analysis. Total proteins were extracted from atria and from hypothalami of both strains. To test the ex vivo processing of atrial pro-ANP, 20 µg of atrial extract from both strains was processed as described previously and analyzed through Western blotting. Protein extracts from hypothalami were also analyzed with Western blotting.

In Vitro Functional Studies of ANP Coding Mutation

AtT-20 cells were transfected with the ANP GC-A cDNA construct. Two days after transfection, they were stimulated with supernatants from cells transfected in parallel with either the ANP SHR or SHRSP construct. Thus, cGMP was collected as described previously and measured with a radioimmunoassay kit (Amersham Corp). The ANP levels of the supernatants used as stimulants were measured as described previously.

Functional Characterization of ANP 5′ End Mutation

A 737-bp fragment of the 5′ URS of ANP (position −683 to +54) was amplified from genomic DNA of both strains, inserted into the pCAT enhancer vector (Promega), and used for transient transfection of primary rat endothelial cells along with the pSVβ-galactosidase construct (Promega). At 30 hours after transfection, the chloramphenicol acetyltransferase (CAT) assay was performed as described previously.

In Vivo Regulation of ANP and BNP Genes

Total RNA was extracted from the brains and atria of rats from both strains that were maintained on either a regular (n = 12) or a Japanese +1% NaCl (n = 12) diet. To detect brain ANP and BNP mRNAs and atria ANP mRNAs, we used an RNase protection assay; [α-32P]UTP antisense RNA fragments were prepared with the use of a Maxiscript kit (Ambion) from ANP, BNP, and β-actin cDNAs, respectively. The RNase protection assay was performed as reported previously. Plasma ANP levels were also measured.

Statistical Analysis

Values for ANP mRNA, plasma ANP levels, cGMP levels, and CAT fold induction are reported as mean ± SEM. Comparisons between groups were performed with one-way ANOVA followed by non-parametric post hoc tests. A value of P < 0.05 was considered statistically significant.

Results

Sequence Analysis

Sequence analysis of the ANP cDNA revealed a G→A substitution in the SHRSP strain at position 1125, within exon 2. As a result, a glycine at position 100 of SHR prepro-ANP is replaced by a serine in the SHRSP. Because this single–base pair substitution abolishes the recognition site for the BstUI enzyme, we were able to confirm it with a PCR-RFLP assay, followed by agarose gel electrophoresis (Figure 1).

The analysis of the 5′ flanking region of ANP revealed a C→A transposition localized at position −463 from the CAP site, changing a putative PE-A-2, polyoma enhancer, binding site from 3'-GCCCGCA-5' in SHR to 3'-GCACCCA-5' in SHRSP. This mutation was clearly confirmed by SSCP (Figure 2). No other sequence difference was found between the two strains.

Comparative sequence analysis of the coding region of BNP failed to show any difference between the two strains.

Protein Analysis

A typical result of the Western blot analysis of the cellular extracts from COS-7 and AtT-20 cells, transfected with either the SHR- or the SHRSP-derived ANP cDNA, is shown in Figure 3A. These experiments provided evidence of a differential processing of the two translational products, depending on their strain of origin. In particular, although the SHR construct yielded predominantly the unprocessed ∼17-kDa protein, with some fully processed ANP, the...
SHRSP-derived construct resulted in the production of a partially processed peptide of ≈6.5 kDa and of some fully processed ANP. Consistently, the analysis of the ex vivo processing of atrial pro-ANP, which confirmed the production of a 6.5-kDa peptide in SHRSP only, provided further evidence of interstrain differences (Figure 3B). Of note, both the in vitro and ex vivo differential processing of the SHRSP pro-ANP peptide occurred despite an identical length of the ANP mRNA in transfected cells and in tissues, as shown by Northern blotting and reverse transcription-PCR (Figure 4), and despite an identical length of the atrial pro-ANP peptide (Figure 3B).

Due to the very limited amount of the whole brain ANP, we chose to consider the ANP peptide forms present in the hypothalamus, where the peptide is relatively more abundant than elsewhere, and we found evidence for differential profiles of peptides between the two strains (Figure 5). Remarkably, the observed processing patterns resembled those seen in the in vitro and ex vivo experiments, although the sites of cleavage used for processing are known to differ in brain and atrial tissues.28

**Functional Studies of Coding Mutation**

Transfection of AtT-20 cells with the rat GC-A receptor cDNA and their subsequent stimulation with the processed pro-ANP forms, produced by cells transfected in parallel with either the SHR or the SHRSP ANP cDNA construct, resulted in a statistically significantly higher cGMP production after stimulation with the supernatant obtained from SHRSP ANP-transfected cells. In particular, after normalization for the ANP concentration of the supernatants, the cGMP-to-ANP ratio (average of 6 independent experiments) was 3.8±0.4 and 5.1±0.4 with SHR and SHRSP ANP stimulation, respectively (P<0.05 versus SHR).

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**Figure 1.** Sequence analysis of site containing coding mutation at position 1125 of ANP in SHR (A) and SHRSP (B). Arrow indicates G→A substitution that occurs in SHRSP. Right, gel shows BstU1-RFLP analysis used to detect this mutation. Lane 1 indicates undigested; and lanes 2, 3, and 4, digested SHRSP samples; lane 5, undigested; and lanes 6, 7, and 8, digested SHR samples.

**Figure 2.** Sequence analysis of site containing regulatory mutation at position –463 of 5’ end of ANP in SHR (A) and SHRSP (B). Arrow indicates G→A substitution that occurs in SHRSP. Right, gel shows SSCP analysis used to detect this mutation. Lanes 1, 2, and 3 indicate SHR samples; lanes 4, 5, and 6, SHRSP samples; and lanes 7, 8, and 9, SHR/SHRSP heterozygous samples.
Analysis of 5’ End Mutation

The activity of the ANP promoter, as determined with the CAT assay of 4 independent experiments, was significantly lower in endothelial cells transfected with the construct containing the SHRSP variant at position -463 compared with the SHR construct (-50%, P<0.05 versus SHR; Figure 6). Preliminary observations in primary rat cardiomyocyte cells are consistent with the findings in endothelial cells.

In Vivo Regulation of ANP and BNP Genes

The expression of ANP was significantly reduced in the cerebral tissue of SHRSP compared with SHR (Figure 7) for both regular and stroke-promoting diets (3-fold lower, P<0.0001). In contrast, atrial expression of ANP was comparable in the two strains (Figure 8). Consistently, because cardiac atria largely represent the major source of circulating ANP, we observed comparable plasma ANP levels in SHR and SHRSP for both dietary regimens: 419±45 versus 346±73 pg/mL (regular diet) and 326±53 versus 287±48 pg/mL (Japanese diet) in SHR and SHRSP, respectively (P=NS).

Of note, measured plasma ANP levels are not indicative of differential processing of the propeptide in the two strains due to the fact that the radioimmunoassay antibody (raised against the final 28–amino acid peptide) is not predicted to differentiate between processed and unprocessed forms.

No differences were found between the two strains with regard to BNP expression.
In a previous study in the SHRSP/SHR F2 intercross, we localized a stroke-relevant gene, \textit{STR2}, within an area of rat chromosome 5 that also carries the genes encoding ANP and BNP.\textsuperscript{12} Both genes colocalize with the peak of linkage of this quantitative trait locus. The known biological cardiovascular properties of these two peptides,\textsuperscript{12–14} as well as their localization in the brain,\textsuperscript{15,16} made them legitimate candidate genes. In addition, the availability of both rat sequence data and rat-specific antibodies facilitated our task of interrogating the possible contributory role of these genes into the higher susceptibility to stroke in the model studied. Although the present findings do not permit us to conclude that the ANP gene is causally linked to stroke in the SHRSP, the observation of altered structure, regulation, and function of ANP is certainly consistent with the hypothesis that this peptide may modulate susceptibility to acute cerebrovascular accidents in our animal model.

In the present experiments, we demonstrate sequence differences between SHR and SHRSP in both the regulatory and the coding regions of ANP. Furthermore, we provide evidence that these differences are associated with both differential regulation and differential processing of ANP, with the latter likely related to predicted structural differences. Furthermore, the lower levels of expression found in SHRSP brains are consistent with the results of promoter studies with the CAT system, in which the SHRSP-derived construct showed decreased activity. Likewise, the results of a number of different approaches, aimed at the study of the resultant peptides, consistently demonstrated differential, typical patterns of processing that are characterized by an accumulation of an intermediate-size peptide from SHRSP transcripts and of larger peptides from SHR transcripts in both transfection experiments and ex vivo observations. No indication for differential length or stability of the ANP message was observed. Last, our cGMP data indicate that the differentially processed SHRSP ANP is also associated with altered biological activity. The latter finding could be explained by the additional intermediate-size peptide or by a different receptor-binding process of ANP products in SHRSP.

A large body of experimental work has shown the presence of ANP in the central nervous system,\textsuperscript{18–20} particularly in the hypothalamus and the anteroventral region of the third ventricle—areas that are critical for body fluid regulation and circulatory control. Brain ANP levels have been previously investigated in the SHR strain and compared with its corresponding normotensive control, the Wistar-Kyoto (WKY) rat.\textsuperscript{29–32} In those studies, a role was postulated for ANP in the development of hypertension. Our data demonstrate substantially lower brain ANP expression in the SHRSP compared with the SHR in the absence of any difference in blood pressure.\textsuperscript{12} Given the facts that the SHRSP (low expression) variant of ANP has been previously linked to a protective effect toward stroke and that recent experiments have demonstrated a relation between stroke and expression of brain adrenomedullin, another potent vasorelaxant peptide, in a model of surgically induced cerebral infarction,\textsuperscript{33} which was worsened by the administration of this peptide, it is intriguing to speculate about a stroke-promoting effect of central nervous system vasodilatation.

The high degree of evolutionary conservation that the glycine at position 100 of ANP shows suggests that amino acid substitution at this position may well affect the translational product. The differential processing, as well as the differential cGMP activity observed for the two transcripts, is

**Figure 7.** RNase protection assay showing brain ANP mRNA in SHR (lanes 1 through 3) and in SHRSP (lanes 4 through 6) for regular (A) and Japanese-style (B) diets. Samples 7 represent heart mRNA.

**Figure 8.** ANP expression in atria (top; \(n=10\) for each experimental group) and brain (bottom; \(n=12\) for each experimental group) from SHR (open column) and SHRSP (filled column) for both regular and stroke-promoting diets. Columns represent ANP mRNA levels after normalization by corresponding \(\beta\)-actin levels. ***\(P<0.0001\) vs SHRSP.
consistent with the putative biological relevance of an amino acid that is very well conserved among species.

A recent study that examines the possible role of ANP in stroke in the rat and fails to find evidence for such a contribution is actually quite consistent with our observations: in this study, SHRSP and WKY rats subjected to middle cerebral artery occlusion showed no differences in brain ANP expression. Of note, there was no difference between the ANP genes of the two strains used with regard to sequence, structure, and functional parameters, as demonstrated previously. An earlier, erroneous interpretation that stroke in an intercross of SHRSP and WKY rats was linked to the ANP locus was indeed corrected by additional data on chromosomal localization of ANP in this recent report.

Although the present data support the notion that ANP may indeed be a candidate gene for contribution to stroke given its localization within the stroke-linked locus 7STR2, final validation of this result must be based on congenic and positional cloning experiments, which are in progress. It is of interest, however, that we were recently able to show that an allelic variant of ANP is associated with a 2-fold risk for stroke in humans. Thus, data from studies in both animals and humans favor further exploration of what seems to be a significant contributory or modulatory role for genetic variants of ANP in the pathogenesis of stroke.

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