The Gene for the Atrial Natriuretic Factor Is Expressed in the Aortic Arch

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SUMMARY The gene for atrial natriuretic factor is expressed within the adventitial cells of the rat aortic arch. Atrial natriuretic factor transcripts, similar in overall size (1100–1200 nucleotides) and 5'-termini to those found in the atria, were identified in the arch. Much lower levels (~10–20%) of these transcripts were present in distal thoracic aorta. Atrial natriuretic factor peptide was localized by immunocytochemistry to the adventitia of the arch in regions thought to harbor the aortic baroreceptors. These data suggest a previously unsuspected role for the peptide in regulating systemic blood pressure through the baroreceptor reflex. (Hypertension 9: 103–106, 1987)

KEY WORDS • atrial natriuretic factor • genetic expression • aortic baroreceptors

THE atrial natriuretic factor (ANF) represents a peptide (or a collection of peptides) with potent effects on the cardiovascular system. As a vasorelaxant,1 natriuretic diuretic,2 and antagonist of the renin-angiotensin-aldosterone axis,3,4 it represents an endogenous defense against volume expansion and hypertension. ANF accumulates in high concentrations within the cardiocytes of the cardiac atria and is secreted into the systemic circulation in response to increased intravascular volume.5 Recently, ANF has also been localized by immunocytochemistry to several areas within the central nervous system, including the hypothalamus, that are thought to be involved in the maintenance of water and electrolyte balance and cardiovascular homeostasis.6,7 We sought to determine whether ANF might be present in those structures outside the central nervous system that are known to play a key role in regulating systemic arterial pressure, specifically, the baroreceptors of the aortic arch.

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Materials and Methods

For RNA blot–hybridization analysis, tissues were excised from approximately 40 Sprague-Dawley rats (weight, 250–300 g; Bantin Laboratories) and immediately frozen in liquid nitrogen until processed. The aortic arch was dissected from a point slightly superior to its exit from the left ventricle to a point approximately 3 to 4 mm distal to the left subclavian artery. Segments (2–3 mm) of the major arterial trunks were left attached to the specimens. "Thoracic aorta" represented the 10 to 15 mm of aorta immediately above the diaphragm. A segment of aorta at least 4 to 5 mm long separated the arch and distal thoracic specimens. RNA was isolated according to the technique described by Cathala et al.8 and denatured in glyoxal as described by Thomas.9 Following size fractionation of total RNA from atria, aortic arch, or distal thoracic aorta on an 1.0% agarose gel and transfer to nitrocellulose filters, hybridization was performed using a rat ANF complementary DNA (cDNA) probe radiolabeled by nick-translation synthesis.10

S, nuclease analyses of ANF transcripts were performed employing an 828 bp EcoRI-BglII rat ANF (rANF) genomic fragment that spans the putative 5' end of the mature rANF transcript. RNA from atria, aortic arch, or thoracic aorta was mixed with approximately 10⁶ cpm of the double-stranded probe radio-labeled at the 5' position.11 The mixture was resuspended in 30 µl of 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide and heated at 85°C for 5
minutes. Subsequent hybridization was performed at 55°C for 24 hours. S1 nuclease digestion was performed in 300 μl of S1 buffer (300 mM NaCl; 300 mM sodium acetate, pH 4.5; 3 mM ZnSO4; salmon sperm DNA, 25 μg/ml) plus 10 μg of S1 nuclease (Bethesda Research Laboratories, Bethesda, MD, USA)/μg RNA at 25°C for 90 minutes. Protected fragments were separated on a 6% polyacrylamide gel containing 8 M urea.

For primer extension analysis, a single-stranded primer (~500 pg) corresponding exactly to a 24-nucleotide segment of the ANF cDNA in the 5’ untranslated region of the gene (nucleotides 33–56) was labeled with phosphorus-32 at its 5’ terminus with polynucleotide kinase and hybridized with 5 μg of atrial or 10 μg of aortic arch or distal thoracic aorta RNA in 10 mM Tris HCl, pH 7.5, and 1 mM EDTA at 50°C for 2 hours. The temperature was then decreased to 45°C, and 15 U of reverse transcriptase in 10 mM Tris HCl, pH 8.0, and 10 mM MgCl2, was added together with all four deoxyribonucleotide triphosphates (250 μM), 5 mM dithiothreitol, and 40 U RNasin (Promega Biotech, Madison, WI, USA); the reaction was continued for 30 minutes. Products were separated on a 6% polyacrylamide gel containing 8 M urea.

Immunocytochemical localization of ANF peptide was performed using antisera directed against the synthetic peptide. Male Sprague-Dawley rats (weight, 200–300 g) were anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg body weight). A cannula was inserted into the aorta through the left ventricle, and the animal was perfused with 50 ml of saline followed by 250 ml of Bouin-Holland sublimate. The tissues to be examined were dissected free and postfixed in the same fixative overnight. The tissues were then rinsed in water, dehydrated in graded alcohols, cleared in xylene, and embedded in Paraplast (Sherwood, St. Louis, MO, USA). Five-micrometer coronal sections were cut and collected on gelatinized slides. Immunocytochemical staining was performed as previously described according to the avidin-biotin complex method. The antiserum used was generated in rabbit against the human ANF (4—28) molecule. This antiserum was fully reactive with rANF (4-28) but failed to react with ANF peptides lacking the phenylalanine-tyrosine-carboxyterminal tripeptide. The ANF antibody was used at a 1:1,000 dilution. Method specificity was tested by confirming the disappearance of the staining when the primary antiserum was used at increasing dilutions. Antiserum specificity was tested by preincubating the antiserum (at a 1:1,000 dilution) with the ANF (4—28) peptide, at a final concentration of 1 μg/ml, which effectively eliminated the staining.

All procedures involving use of laboratory animals were performed in accordance with institutional guidelines.

Results

Blot-hybridization analysis revealed detectable ANF messenger RNA (mRNA) in atria, aortic arch, and distal thoracic aorta. As shown in Figure 1A, the aortic arch harbored a transcript that hybridized to the ANF cDNA and was approximately the same size (~1100–1200 nucleotides) as the mature atrial transcript. The transcript was much less abundant in the aortic arch than in the atria, while the distal thoracic aorta demonstrated even lower ANF mRNA levels.

To determine whether these transcripts arose from a common RNA polymerase II promoter structure, an S1 nuclease analysis of the transcripts was done employing a 5’-labeled EcoRI-BgIII fragment that spans the 5’ end of the rANF gene. As shown in Figure 1B, RNA from aortic arch as well as from the atria protected a fragment approximately 185 to 190 nucleotides in length. This mapped to a position that was virtually identical to the presumptive ANF mRNA start site as described by Argentin et al. A small amount of a similarly protected fragment was present in the distal thoracic aorta, although the levels were much lower than those found in the aortic arch. Analysis of RNA extracted from abdominal aorta revealed ANF mRNA levels equal to or less than those found in the distal thoracic aorta, while RNA isolated from the carotid sinus had substantially lower ANF transcript levels (data not shown). In addition to the major protected fragment at 185 to 190 nucleotides, there were several additional bands at 220, 240, and 270 nucleotides in atrial RNA. These transcripts have also been identified by primer-extension analysis (D.G. Gardner, unpublished observations, 1986), indicating that they represent bona fide ANF gene products. The largest transcript was approximately 20 to 30 base pairs downstream from an adenine thymidine-rich region, which could function as a weak RNA polymerase II promoter structure; the intermediate bands may result from the same or similar vestigial promoter(s), or they could represent partially processed products of the larger transcript. The finding that similar-sized fragments can be identified in RNA from the aortic arch (Lane 2) as well as from the distal aorta (Lane 3) suggests the use of similar transcription start sites in these tissues. The more prominent bands present in all three lanes at 320 nucleotides and larger also were present in a control S1 digestion containing no RNA and, therefore, probably represent incomplete digestion products of the S1 nuclease reaction; the band at the very top of each lane represents reannealed probe (data not shown).

The locations of the 5’ termini were confirmed by primer extension analysis (Figure 1C). In the atria as well as the aortic arch, total RNA primed fragments 72 to 76 nucleotides in length identifying 5’ termini approximately 20 to 25 nucleotides downstream from the TATAAAAA sequence thought to dictate the transcription start site of the rANF gene. This position overlies that determined by S1 analysis, as already shown. No primer-extended products in the 70 to 80 nucleotide range could be identified in RNA from the distal aorta.

Similarly, immunoreactive ANF peptides were identified in extracts of aortic arch. We used a modification of the protein extraction technique of Lang et al. and an rANF radioimmunoassay developed in our laboratory, and we found that the aortic arch contained
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FIGURE 1. A. Blot-hybridization analysis showing 40 μg (Lane 1) or 4 μg (Lane 2) of total RNA from the abdominal aorta, 40 μg (Lane 3) or 4 μg (Lane 4) of RNA from the aortic arch, and 5 μg (Lane 5) or 0.5 μg (Lane 6) of RNA from the cardiac atria. Samples were size-fractionated on 1% agarose gel and blot-hybridized to a 32P-radiolabeled ANF cDNA probe, as described in the text. Major transcripts were 1100 to 1200 nucleotides long, as determined by comparison with independently run DNA size markers. Film was exposed for 96 hours. B. S1 nuclease analysis showing 5 μg of total RNA from atria (Lane 1), 6 μg from aortic arch (Lane 2), or 10 μg from thoracic aorta (Lane 3). Samples were hybridized with a 32P-radiolabeled 828-base pair EcoRI-BgIII fragment that spans the 5' end of rat ANF gene. 14 The hybrids were treated with S1 nuclease, and the protected fragments were separated on a denaturing 6% polyacrylamide gel. Vertical numbers represent DNA size markers (Lane 4) in base pairs. C. Primer extension analysis showing 5 μg of total RNA from atria (Lane 1), 6 μg from aortic arch (Lane 2), or 10 μg from thoracic aorta (Lane 3). Samples were hybridized to a 32P-radiolabeled 24-nucleotide DNA strand complementary to the 5'-untranslated region of the rat ANF gene. This fragment was extended using reverse transcriptase, and the products were size-fractionated on a denaturing 5% polyacrylamide gel. Vertical markers represent DNA size markers (Lane 4) in base pairs.

4038 ± 1404 (SD) pg of ANF/mg soluble protein (n = 3). This represents approximately 2% of the ANF immunoreactivity detected in atria subjected to an identical extraction procedure and approximately 13 times the activity found in liver, a tissue not thought to express the ANF gene at a significant level (D.G. Gardner, unpublished observation, 1986).

Immunocytochemistry was performed to define more precisely the site of ANF gene expression in the aortic arch. As shown in Figure 2, immunoreactive ANF was readily detected in the adventitia of the arch, a finding that has been reproduced in four additional animals. No activity was detected along the endothelial surface, in the muscularis of the arch, or in aortic sections taken from the lower thoracic and abdominal aorta. No staining was observed when the antibody, used at a 1:1000 dilution, was preincubated with ANF, 1 μg/ml At a higher magnification this material appeared to be confined to disorganized clusters of tangled structures reminiscent of those thought to represent the arterial baroreceptors. 15 Positive immunoreactivity was especially dense along the bifurcation of the major arterial trunks arising from the arch, again mirroring the distribution of the baroreceptors.

Discussion

The adventitial concentration of immunoreactivity plus the localization to bifurcations of the major vessels strongly suggests that these ANF-containing cells either represent the aortic baroreceptors themselves or lie in close proximity to the baroreceptors. The detec-

FIGURE 2. A. Immunocytochemical localization of the ANF peptides. Coronal section of the aortic arch. The bulk of the ANF immunoreactivity (arrows) is located at the bifurcation of the aorta (aor) and the common brachio-carotid artery (car). (× 25; bar = 135 μm.) B. Coronal section of the aortic arch at the level of the bifurcation of the aorta and the common brachio-carotid artery. The ANF immunoreactivity (arrows) is detectable in the adventitial portion of the vascular wall. (× 40; bar = 80 μm.) C. Coronal section of the aortic arch. ANF immunoreactivity confined to poorly defined elongated elements in the adventitia. No immunoreactivity is detectable within the muscularis of the vessel. (× 63; bar = 50 μm.) D. Coronal section of the distal aorta. No ANF immunoreactivity is detectable. (× 40; bar = 80 μm.)
tion of ANF mRNA transcripts as well as the adventi-
tial concentration of immunoreactivity (i.e., away
from the circulating plasma) provides support for the
argument that aortic arch ANF results from synthesis
of the protein in this tissue rather than from binding of
the peptide to surface, or subsequently internalized,
ANF receptors.

The function of ANF in the aortic arch is unknown.
Our findings raise the possibility that ANF may have a
paracrine or autocrine role in mediating or modulating
the baroreceptor reflex. The afferent limb of this reflex
originates in the sensory fibers of the baroreceptors
themselves, traverses the vagus nerves to the brain-
stem, and terminates in the nuclei of the tractus solitar-
us. Interneurons in these nuclei relay information to
the vasomotor center as well as to the cardioinhibitory
center located in the brainstem. The net result is a
reduction in the tonic discharge of the vasoconstrictor
nerves, producing vasodilatation, a decrease in blood
pressure, and a centrally mediated bradycardia.15 Inter-
estingly, several of these effects mirror those seen fol-
lowing administration of exogenous ANF in vivo,16
and at least one group has reported blunting of the
response to atrial extracts following vagotomy.17 This
response raises the intriguing possibility of a neurally
mediated ANF function that acts in parallel with its
hormonal activity.

The presence of ANF mRNA in more distal aortic
tissues, as well as in the carotid sinus, indicates that
vascular expression of the peptides is not confined to
the arch. This may represent coincidental low level
expression of the ANF gene, which has little physio-
logical importance, or more likely, expression de-
signated to subserve a similar physiological function but
amplified to a highly differentiated form within the
arch. The data also suggest that whatever function
ANF subserves in the arch, it probably is not employed
in the carotid sinus, the other major baroreceptor focus, since much less ANF
mRNA could be detected in that organ (data not
shown).

The fact that we found ANF mRNA but not immu-
noreactive peptides in distal aorta may be explained by
differences in the sensitivities of the respective tech-
niques employed for analysis (i.e., sensitivity of the
RNA measurements may be much better than those for
the immunocytochemical analysis). Alternatively, it
could reflect differences in the peptide storage capac-
ties of these tissues, a situation in which new synthesis
might have a minimal impact on the immunocyto-
chemical signal.

In summary, ANF appears to be synthesized in or
near the baroreceptors of the aortic arch. If ANF is
involved in mediating or regulating the baroreceptor
reflex, then ANF may serve a dual role as a phys-
iological regulator, participating in the acute neuro-
genomic response as well as in the more chronic hor-
monal response to volume expansion and systemic
hypertension.

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