Structure and Physiological Actions of Rat Atrial Natriuretic Factor

TADASHI INAGAMI, KUNIO S. MISONO, HIROSHI FUKUMI, MASATOSHI MAKI, ISSEI TANAKA, RYOICHI TAKAYANAGI, TERUAKI IMADA, ROBERT T. GRAMMER, MITSUHIDE NARUSE, KIYOKO NARUSE, KAILASH N. PANDEY, MARC PARMENTIER, MINORU YASUJIMA, AND KEISHI ABE

SUMMARY Natriuretic substances were purified from rat atrium (atrial natriuretic factor, ANF) and were shown to be identical with the inhibitor of norepinephrine-induced contraction of smooth muscle. Four native forms were isolated and their amino acid sequences were determined. The presence of a high-molecular-weight prohormone was shown. Complementary DNA (cDNA) encoding for the precursor was cloned and used to deduce the amino acid sequence of the prohormone. Genomic DNA for ANF was cloned and two introns were found. Several ANF peptides were synthesized. Structure-function studies showed that the ring structure was essential for the activity. Antibodies produced against the synthetic 25-amino acid residue ANF were used to develop a radioimmunoassay. The presence of ANF in rat plasma demonstrated that ANF is a circulating hormone. ANF was also found in the hypothalamus of rats. The ANF in plasma was found to be a low-molecular form, whereas that in atria and hypothalamus consisted of both the high-molecular-weight precursor and low-molecular-weight active ANF. The presence of messenger RNA for ANF was determined using ANF cDNA as a probe and was considered as evidence for ANF synthesis in the brain, atrium, and ventricles. ANF was shown to be released from the brain. ANF administered intracerebroventricularly was shown to inhibit angiotensin II and thirst-induced dipsogenesis. In vitro and in vivo experiments showed ANF inhibits release of vasopressin from posterior pituitary and renin from the kidneys. The hypotensive effect of ANF was examined at various doses. At a subpharmacological dose, ANF seems to function as a buffer against sudden surge of blood pressure induced by vasopressor substances. Plasma ANF concentration was determined under various conditions affecting blood pressure, and evidence was obtained indicating that increased blood pressure stimulates ANF release. Two types of ANF receptors were purified. Specific peptidases responsible for the conversion of proANF to circulating ANF were isolated. (Hypertension 10 [Suppl T]: I-113-I-117, 1987)

KEY WORDS • atrial natriuretic factor • purification • complementary DNA • renin release • vasopressin release

EXTRACT of the atria of the heart has been shown to elicit potent natriuretic and diuretic activities (atrial natriuretic factor, ANF), and to inhibit the contraction of smooth muscle induced by norepinephrine, angiotensin II, or potassium.

Purification of ANF Peptides
We purified these apparently unrelated activities to homogeneity by several steps of high-performance liquid chromatography (HPLC) in 1983. The natriuretic and smooth muscle relaxant activities were shown to coelute inseparably throughout these purification steps. Four peptides with 25 to 35-amino acid residues were isolated from rat atria and their structures were determined.

Structure of ANF Peptides
The four peptides purified by us from rat atria, ANF-(92-126), ANF-(96-126), ANF-(96-125), and ANF-(102-126) showed similar ID values in inhibiting the norepinephrine-induced contraction of rabbit aortic strip. The smallest of them was the 25-amino...
acid residue peptide ANF IV-(102-126). On the other hand, 21-residue ANF-(103-123) (atriopeptin I) showed a greatly reduced inhibition of norepinephrine-induced contraction of an aortic strip. This reduction amounted to two orders of magnitude.

Cleavage of the disulfide bridge by reduction and carboxymethylation completely destroys the activity. Similarly, the selective cleavage of an aspartyl peptide bond in the ring structure by staphylococcal protease V8 completely destroys the activity.

**Structure of ANF Precursor Deduced from Its cDNA Structure**

In general, peptide hormones are formed from larger prohormones by proteolytic cleavage. Observation of higher-molecular-weight ANF and a series of low-molecular-weight ANFs with different molecular sizes indicated the presence of precursors of ANF.

We cloned ANF complementary DNA (cDNA) using as probes synthetic mixed oligodeoxynucleotides based on the amino acid sequence of the active peptide hormone. A full-length cDNA insert was identified in plasmids in transfected *Escherichia coli*, and its deoxynucleotide sequence of the cDNA insert was determined. It contained 786 nucleotides with 63 nucleotides in the 5'-noncoding region, and 363 nucleotides in the 3'-noncoding region. The coding region consisted of 152 amino acid residues with a 24-amino acid residue signal sequence. The carboxyl terminal amino acid sequence completely agreed with those determined by Edman degradation. In addition, an Arg-Arg sequence was found attached to the carboxyl terminal of tyrosyl residue.

Using cDNA as probe, we cloned human ANF genomic DNA from a library of human genomic DNA. The genomic DNA for ANF was found to contain two introns, one between the triplets coding for Lys-41 and Asn-42, and the other in the carboxyl terminal region. The genomic DNAs for human and mouse ANF have been cloned by several groups.

**Chemical Synthesis of ANF Peptides**

The solid phase synthesis method was used to obtain ANF II (31 residues), ANF III (30 residues), ANF IV (25 residues), ANF IV with additional Arg-Arg attached to its carboxyl terminal, and the 17–amino acid ring without tails. These peptides showed properties identical with the corresponding natural peptide.

This macrocyclic peptide with the 17–amino acid residue ring structure was found to possess considerable natriuretic activity and the ability to relax carbachol-induced contraction of chick rectum, although it is two orders of magnitude less efficient than ANF peptides containing more than 25 amino acid residues in relaxing norepinephrine-induced contraction of rabbit aortic smooth muscle.

**Conversion of Pro-ANF to Active ANF**

While the low-molecular-weight ANF was isolated from rat atrium in the early stage of ANF studies, more recent studies employing rat atria boiled in 0.1 N acetic acid immediately after the excision of the tissues revealed that practically all of the ANF in the atria is of the high-molecular-weight form (determined by gel filtration chromatography on BioGel P-10 followed by reversed phase high-performance liquid chromatography on ODS column). On the other hand, the circulating form in plasma was found to elute mostly in a single peak with a low molecular weight. These findings indicate that the high-molecular-weight form is converted to the circulating form at the time of secretion. In later studies the circulating form of ANF in the rat was isolated and identified as 28–amino acid ANF-(99-126) by Schwartz et al. and Thibault et al. We localized the enzyme responsible for the generation of ANF-(99-126) in the membrane fraction of atrial homogenate, purified it, and identified it as serine protease.

**Radioimmunoassay of ANF**

Synthetic ANF coupled to bovine thyroglobulin by glutaraldehyde was used as immunogen to elicit the production of anti-ANF antibody in rabbits. These antibodies were used to develop a radioimmunoassay of ANF and to identify ANF in rat tissues immunohistochemically.

Radioimmunoassay of rat and human ANF developed in our laboratory permitted determination of 3 to 5 pg of ANF. The extraction was essential for the assay. Without the extraction, ANF concentration values were five to ten times greater than the extracted plasma.

The assay method was also applied to the determination of plasma and atrial, ventricular, and brain concentrations of ANF. Rat plasma collected under pentobarbital anesthesia or decapitation gave similar plasma concentrations of 20 to 40 fmol/ml (60–120 pg/ml). The presence of ANF in blood had not been established before this study. This finding established ANF as a circulating hormone.

We investigated the content of ANF messenger RNA (mRNA) by hybridization using ANF cDNA labeled with ^32P by nick-translation in the atria and ventricles. Levels of ANF mRNA were not increased in atrium after 2 weeks of salt loading with 1.8% NaCl in drinking water.

**ANF in Plasma**

Levels of ANF in plasma were found to be elevated in hypertensive rats. In spontaneously hypertensive rats (SHR) the level steadily increased with age and blood pressure from approximately 100 pg/ml in 3-week-old normotensive SHR to 450 pg/ml in 15-week-old rats with systolic pressure greater than 165 mm Hg. In the model of pulmonary hypertension generated by the treatment of rats under hypoxic conditions for 21 weeks, plasma ANF was elevated from 101 to 238 pg/ml. A similar increase from 117 to 238 pg/ml was observed during the development of salt-induced hypertension in Dahl salt-sensitive rats. These findings suggested that plasma ANF is elevated as a secondary result of elevation in blood pressure, presumably due to distention of the atria.
ANF in the Brain

The presence of ANF in rat hypothalamic and pontine regions was discovered by radioimmunoassay. The storage form of ANF in rat brain was in a low-molecular-weight form(s), with 24 or 25 amino acid residues, according to Shiono et al. This is in marked contrast to the high-molecular-weight form (126 amino acid residues) stored in the rat atria.

Brain ANF was secretable, for example, in response to potassium-induced depolarization. This secretion was dependent on calcium. Intracerebroventricularly administered ANF clearly suppressed the thirst- or angiotensin II (ANG II)-induced dipsogenesis by approximately 35%, in agreement with observations by Antunes-Rodrigues et al. and Nakamura et al.

Endocrine Functions of ANF

ANF was shown to inhibit aldosterone secretion by several groups of investigators. These findings indicated that ANF exhibits a concerted action toward reducing extracellular fluid volume. Vasopressin and angiotensin also play roles in fluid volume regulation. In in vitro superfusion of rat posterior pituitary ANF (10^-10 to 10^-6 M) was found to inhibit vasopressin release at ANF concentrations of 10^-10 M. This observation was confirmed by Januszewicz et al. but is contradictory to an earlier report by them that ANF stimulates vasopressin release from rat neurophysys. The factor was reported to inhibit thirst- or hemorrhage-induced vasopressin release by Samson.

In experiments using rat kidney slices as well as in vivo studies, it was shown that ANF causes a significant decrease in renin release in vitro or lowering of plasma renin activity concomitant with a reduction in cyclic adenosine 3',5'-monophosphate production and an increase in cyclic guanosine 3',5'-monophosphate (cGMP) production.

To assess its physiological roles, attempts were made to eliminate circulating ANF by intravenous administration of an excess of ANF antibodies. Responses elicited were a reduction in diuresis and natriuresis and an increase in plasma renin. No effect on plasma aldosterone or blood pressure was observed.

ANG II is known to facilitate cGMP in target cells and by Waldman et al. that this is due to simulation of the membrane-bound form of guanylate cyclase. While many investigators have demonstrated ANF receptors in various tissues, use of the photoaffinity-labeling ligand azidobenzoyl-ANF permitted Misono et al. to identify ANF receptor in the adrenal capsular cells as a single 135,000-molecular-weight protein with a single high-affinity constant. A similar receptor was identified in mouse testicular Leydig tumor cell line. However, several investigators reported a second type of ANF receptor with a molecular weight in the 60,000 range. Takayanagi et al. using three steps of affinity chromatography, purified two types of ANF affinity, one linked to guanylate cyclase and the other not linked to the enzyme. The latter reacts with 21-amino acid ANF (atriopeptin I) or the 17-residue ring peptide, but the former requires ANF with 25 residues or more.

Effect of ANF on Blood Pressure

The in vitro smooth muscle relaxant activity suggested that ANF would be a vasorelaxant and reduce blood pressure. At a high dose of 10 to 20 μg per rat in bolus it evoked a marked hypotension. At 6 μg the hypotensive response was elicited in SHR, but its effect on normotensive rats was much diminished. The effect of ANF (6 μg/rat in bolus) was most pronounced in increasing renal and adrenal blood flow of SHR.

These results indicate that SHR are more sensitive in hypotensive response to ANF in spite of the elevated plasma ANF and consequent down-regulation of ANF receptors.

At a much reduced subpressor dose of ANF infused at a rate of 150 μg/kg/day, Yasujima et al. found that it completely suppressed the hypertensive effect of norepinephrine infused at a flow rate of 0.36 mg/day in conscious rats. Infusion of ANF also corrected the hypertension induced by the infusion of norepinephrine, which elevated the systolic pressure to 150 mm Hg or higher. At the same infusion rate of ANF, the hypertensive effect of ANG II (900 μg/kg/day) was completely suppressed in rats.

Although plasma ANF levels were not determined in these low-level infusion experiments, the level seems to be closer to physiological conditions. On the other hand, the microgram dose employed to induce hypotension was calculated to raise plasma ANF to a concentration range of 50 ng/ml or greater, which is 500 to 1000 times greater than the normal level. Thus, it is more realistic to consider ANF as a buffering agent that protects the cardiovascular system against a surge of blood pressure caused by sudden activation of the adrenergic or renin-angiotensin system rather than primarily as a hypotensive agent or a regulator of blood pressure. The lack of effect on blood pressure of antisera to ANF also indicates that ANF at physiological concentrations has no direct effect on blood pressure.

ANF Receptor and Guanylate Cyclase

It has been shown by Hamet et al. that ANF increases cellular cGMP in target cells and by Waldman et al. that this is due to simulation of the membrane-bound form of guanylate cyclase. While many investigators have demonstrated ANF receptors in various tissues, use of the photoaffinity-labeling ligand azidobenzoyl-ANF permitted Misono et al. to identify ANF receptor in the adrenal capsular cells as a single 135,000-molecular-weight protein with a single high-affinity constant. A similar receptor was identified in mouse testicular Leydig tumor cell line. However, several investigators reported a second type of ANF receptor with a molecular weight in the 60,000 range. Takayanagi et al. using three steps of affinity chromatography, purified two types of ANF affinity, one linked to guanylate cyclase and the other not linked to the enzyme. The latter reacts with 21-amino acid ANF (atriopeptin I) or the 17-residue ring peptide, but the former requires ANF with 25 residues or more.

In the study of ANF receptors it has been found that ANF receptors in aortic smooth muscles and adrenal capsules are markedly down-regulated in SHR to a level of receptor density that is 40% of that of normotensive Wistar-Kyoto (WKY) strain. This is most likely due to the elevated plasma ANF levels in SHR. As stated above, ANF stimulated particulate guanylate through interaction with ANF receptor. Strangely, cGMP concentrations in the aorta, adrenal cortical cells, anterior pituitary, and medulla oblongata were markedly elevated in SHR compared with WKY in spite of reduced receptor density in the hypertensive rats. These observations indicate that an additional regulatory mechanism may exist between ANF receptor and activation of guanylate cyclase.
Although ANF causes aldosterone secretion and simultaneous elevation of the cGMP level in adrenal zona glomerulosa cells, a sodium nitroprusside-induced increase in cGMP or administration of permeable cGMP analogues does not inhibit aldosterone release. There is a good possibility that ANF signal may be transmitted by a mechanism other than cGMP mediation.

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Hypertension. 1987;10:I113
doi: 10.1161/01.HYP.10.5_Pt_2.I113

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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