Atrial Natriuretic Factor and Transgenic Mice

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Atrial natriuretic factor (ANF) is a peptide hormone that induces potent but transient hypotensive and natriuretic responses on short-term administration. The role of the hormone in long-term cardiovascular regulation has remained elusive in part because of the temporal limitations of long-term infusion models and the complexity of the hormone. The transgenic mouse model was developed that exhibits lifelong elevated plasma ANF levels. These mice are chronically hypotensive, with arterial pressures averaging 20 to 30 mm Hg less than those observed in nontransgenic siblings. In contrast, no obvious natriuretic or diuretic phenotype was observed in transgenic animals housed in metabolic cages. Thus, the mice adequately compensate for the renal effects but not the hemodynamic effects of the hormone. The ANF transgenic mice provide a tractable model system with which to study the consequences of long-term alterations of ANF expression in vivo. (Hypertension. 1993;22:634-639.)

KEY WORDS • atrial natriuretic factor • mice, transgenic

Molecular Biology of Atrial Natriuretic Factor

After the discovery of ANF in 1981, 25 years after volume-expansion studies suggested that increased atrial pressure or stretch leads to diuresis and natriuresis, the molecular characterization proceeded rapidly (for review, see References 1 through 4). The structural organization of the ANF gene has been determined from several species. The gene is divided between three exons and two introns and encodes a mature ANF transcript that is approximately 900 bases in length. The primary source of ANF synthesis is the cardiac atria, where ANF transcripts have been detected as early as day 8.5 in developing mouse embryos. In contrast, although ANF transcripts are observed in ventricular cardiomyocytes in early development, expression at this site ceases soon after birth. Interestingly, ventricular ANF expression can be induced in adult animals subjected to pressure or volume overload. In addition to the heart, low levels of ANF transcripts have been detected in the central nervous system, aortic arch, thoracic aorta, and pulmonary epithelium.

The human ANF precursor is a 151-amino acid prohormone. Cleavage of a 25-amino acid signal peptide generates pro-ANF (also known as ANFp). The physiologically active portion of the molecule has been localized to the 28 carboxy terminal residues and has a central ring structure formed by a disulfide bridge between cysteine residues 105 and 121. The prohormone is stored in electron-dense secretory granules localized in the perinuclear region of atrial cardiomyocytes. In response to transmural pressure increases, the granule contents are released to the extracellular space. An undetermined cleavage mechanism releases ANFp from the amino terminal fragment during the secretion process.

Short-term Physiological Effects of Atrial Natriuretic Factor

Acutely administered ANF has a wide range of biologic actions. In the cardiovascular system, ANF

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decreases blood pressure, cardiac output, total peripheral resistance, and blood volume. Short-term infusion or bolus injection of ANF produces rapid, sustained, dose-dependent decreases in arterial pressure in the absence of a reflex tachycardia. This occurs in both conscious and anesthetized normotensive and hypertensive animals.²⁻⁴ The fall in cardiac output by short-term administration of ANF has been ascribed to both direct and indirect effects. Direct regulation occurs via decreased contractility, whereas a reduction in the central venous or right atrial pressure indirectly affects cardiac output by decreasing preload. The effects of ANF on total peripheral resistance were not consistent. These discordant results might reflect differences in resting vascular and autonomic tone in the various experimental models used. Decreases in plasma volume after ANF administration are due at least in part to a shift in fluid from the intravascular to the interstitial compartment caused by an increase in intrinsic capillary hydraulic permeability.

In the renal system, ANF administration increases excretion of fluid and electrolytes. This natriuretic and diuretic response is due to a combination of increased glomerular filtration rate (GFR), modulation of renal vascular resistance, increased sodium excretion by inner medullary collection duct cells, and decreased inner medullary hypertonicity. In the endocrine system, ANF is a powerful inhibitor of the renin-angiotensin-aldosterone system at both the hormonal secretion and target organ levels. Specifically, short-term ANF administration inhibits renin secretion from juxtaglomerular cells, thereby decreasing plasma renin activity. ANF also inhibits angiotensin II-induced vasconstrictor and pressor responses, inhibits endocrine vasopressin and corticotropin release, and inhibits aldosterone production and secretion from the adrenal gland. Additionally, ANF inhibits electrically stimulated norepinephrine release from adrenergic nerve endings. All of the above short-term effects of ANF in animals are directed toward the reduction of blood pressure and blood volume.

The various systemic actions of ANF are mediated through the GC-A ANF and GC-B ANF receptors, which generate intracellular cGMP in a dose-dependent manner.¹⁰⁻¹² These receptors are unique in that a single protein exhibits the ability to bind ligand at the extracellular domain and to catalyze second messenger production at the intracellular domain. The cytoplasmic domains of the GC-A ANF and GC-B ANF receptors share marked homology.¹²⁻¹³ In contrast, the short cytoplasmic domain of the clearance (C)-ANF receptor is typical of other nutrient transport or clearance receptors.¹¹ Structural differences in the extracellular domains of these three receptors result in functional differences in ligand binding of ANF, the related brain natriuretic peptide and C-type natriuretic peptide, and the synthetic selective C-ANF receptor ligand cANF₄₋₁₀.¹¹⁻¹⁵

Although these short-term effects of ANF are well characterized, the role of the hormone in long-term cardiovascular and renal regulation has been difficult to assess. Techniques to increase blood or tissue ANF concentrations are hindered by its very short plasma half-life (t₁/₂≈20 to 180 seconds). Two major degradative pathways are responsible for the rapid clearance of ANF:²⁻⁴,¹¹⁻¹⁴,¹⁶ The first relies on the high-affinity C-ANF receptor, which is expressed in cell types exposed to a large portion of the cardiac output, as, for example, vascular endothelial cells and renal cortical cells and glomeruli. The absence of physiological activity associated with C-ANF receptor–ligand binding has led to the suggestion that its main function is the clearance of ANF from circulation by receptor-mediated endocytosis.¹¹⁻¹⁴ This suggestion is supported by the observation that administration of agonist, cANF₄₋₁₀, specific for the C-ANF receptor but not the GC-coupled receptors results in systemically elevated levels of endogenous ANF. The increased plasma ANF level is thought to reflect inhibition of clearance by the C-ANF receptor. The second ANF degradative pathway involves neutral endopeptidase 24.11, which disrupts the ring structure of ANF.¹⁶ Pharmacologic agents that block neutral endopeptidase 24.11 also increase circulating ANF levels.

The relatively short half-life of ANF in the circulation has made it difficult to study the long-term role of the hormone in cardiovascular regulation. Nonetheless, several groups have reported on studies with long-term ANF infusions (1 to 3 weeks) in a variety of species. These studies have given conflicting results regarding the ability of ANF to change cardiovascular, renal, and endocrine function.¹⁷⁻²³ Long-term infusions of ANF have been observed to decrease mean arterial pressure in both normotensive¹⁸⁻⁻¹⁹,²¹⁻²³ and hypertensive¹⁷⁻⁻²² animals in several studies. This effect was accompanied by variable changes in cardiac output, total peripheral resistance, intravascular volume, and renal function. In other studies, long-term ANF infusions did not result in sustained hypotension in either normotensive¹⁷ or hypertensive²² animals. Undoubtedly, differences in the methods used in the various studies have contributed to this discrepancy. For example, the effective increase in steady-state plasma ANF concentrations attained as well as the duration of infusion must be considered when these data are evaluated. Despite these discrepancies, it is clear that long-term infusion studies are absolutely required for the evaluation of ANF as a therapeutic agent for hypertension, congestive heart failure, and acute and chronic renal failure.

Transgenic Mice With Chronically Elevated Plasma Atrial Natriuretic Factor

To further evaluate the role of ANF in long-term cardiovascular regulation, our laboratory has generated a transgenic mouse model that exhibits chronically elevated plasma ANF levels.³ These mice, designated TTR-ANF, carry a transgene consisting of the mouse transthyretin (TTR) promoter and the mouse ANF structural gene (Figure, A). Our rationale was that transgene expression would target ANF mRNA synthesis to hepatocytes and that the pro-ANF encoded by these transcripts would be constitutively secreted into the circulation. If successful, this model would provide a steady-state high plasma ANF level by noninvasive means and in the absence of temporal limitations.

The TTR-ANF transgenic mice proved to be an interesting model system with which to assess the consequences of chronically elevated plasma ANF.³ Radioimmunoassays revealed that the transgenic mice had an approximately 10-fold increase in plasma ANF levels compared with their nontransgenic littersmates. Ele-
Structure of atrial natriuretic factor (ANF) transgenes. A: TTR-ANF transgene. The TTR-ANF gene was designed such that the mouse transthyretin (TTR) promoter would target expression of ANF mRNA to the liver. Mouse TTR promoter was isolated from a BALB/cCr genomic library and is localized on a 3-kb EcoRI/Xho II restriction fragment. The Xho II restriction site lies at position +18 in the 5' untranslated leader. Mouse ANF sequences were also isolated from a BALB/cCr genomic library. The promoter and a region of the 5' untranslated leader of the ANF gene were removed by Bal 31 exonuclease, and a Sal I restriction site was introduced at position +41 relative to the transcription initiation site of the native ANF gene. This fragment was ligated into the clone carrying the TTR sequences such that transcripts arising from the TTR promoter would encode prepro-ANF. B: mMHC-ANF transgene. Mouse α-cardiac myosin heavy chain (mMHC) promoter has been used to target ANF expression to ventricular cardiomyocytes. This promoter was previously described by Robbins and coworkers and consists of 4.5 kb of 5' flanking sequence, as well as exons 1 and 2 and a portion of exon 3 (the endogenous MHC initiation codon is not included in the subclone). Studies from the Robbins laboratory have shown that this promoter targets uniform expression throughout the atria and ventricles. This promoter has been fused to the same ANF gene as described above. C: TTR-NTF transgene. The TTR--amino terminal fragment (NTF) transgene is identical to the TTR-ANF transgene with the exception that an in-frame stop codon has been engineered into the ANF sequences at serine 99 of pro-ANF. D: VSMA-ANFR transgene. Mouse vascular smooth muscle α-actin (VSMA) promoter will be used to target expression of an activated ANF receptor (ANFR) to vascular smooth muscle. The promoter consists of 1079 bp of 5' flanking sequence, exon 1, intron 1 (2.2 kb), and 20 nucleotides of exon 2. The VSMA initiation codon is not present in this subclone. This promoter has targeted high levels of chloramphenicol acetyItransferase (CAT) expression in cultured aortic smooth muscle cells but not in HeLa cells. Expression analyses of mice that carry a VSMA-CAT fusion gene indicate that the promoter is specific for vascular smooth muscle (A.R. Strauch, L.J. Field, unpublished observation). GC, guanylate cyclase.
vated plasma ANF levels were detected as early as 3 weeks of age and persisted throughout the life span of the mice. In experiments with conscious instrumented animals, mean arterial blood pressure was approximately 20 to 30 mm Hg lower in the TTR-ANF transgenic mice compared with their nontransgenic littermates. This reduction was also observed in mice anesthetized with both avertin and thiobutabarbital. The TTR-ANF transgenic mice maintained normal heart rates despite the marked hypotensive phenotype, in agreement with the observation that hypotension induced by bolus ANF administration is not accompanied by the expected reflex tachycardia. Perhaps the most surprising observation with the TTR-ANF mice was the absence of a pronounced effect on water and electrolyte balance, despite the presence of hypotension and elevated plasma ANF levels. Plasma sodium and potassium contents, water intake, and urinary excretion of water and electrolytes did not vary markedly between the transgenic mice and their nontransgenic siblings.

In collaboration with Harald Sonnenberg's group at the University of Toronto, the renal response to short-term volume expansion was characterized in the TTR-ANF mice. When extracellular volume was expanded by approximately 25%, water and electrolyte excretion was enhanced in the TTR-ANF mice compared with controls, despite the retention of the blood pressure differential. There were no differences in either GFR or hematocrit between groups before or after short-term volume expansion. Several mechanisms can be evoked to explain these results. First, ANF-induced hypotension and the concomitant decrease in renal perfusion pressure may directly counteract (in part) the natriuretic and diuretic effects of the hormone, thereby maintaining water and electrolyte balance. Second, ANF may shift the normal pressure-natriuresis curve toward lower arterial pressure so that sodium balance is maintained at a reduced blood pressure. Finally, there may be minimal renal desensitization and downregulation of the GC-A ANF receptor in this model.

Results obtained from short-term ANF administration studies provide additional insight into the mechanism by which the TTR-ANF mice may maintain normal GFR in the presence of chronic hypotension and elevated plasma ANF levels. For example, ANF has been shown to significantly dilate the preglomerular arterioles and constrict the postglomerular arterioles in vitro-prepared afferent and efferent arterioles from transgenic mice compared with nontransgenic siblings.

Future Goals

Although it is clear that the elevated plasma ANF in the TTR-ANF mice is responsible for the observed chronic hypotension, the precise mechanisms for this phenotype remain unknown. Interpretation of the model is complicated by the observation that the preponderance of immunoreactive ANF in the transgenic mice is unprocessed prohormone, suggesting that transgenic hepatocytes secrete only pro-ANF and that the implicit processing occurs in the circulation. Accordingly, we are producing additional transgenic models that should help clarify the role of ANF in long-term cardiovascular regulation.
mMHC-ANF Mice

Given the issue of circulating pro-ANF in the TTR-ANF mice described above, we sought to identify a potential cell type in which targeted ANF could be processed. One obvious cell type is the ventricular cardiomyocyte, which is able to secrete processed ANF.30 Thus, we have recently produced a new transgenic model in which the mouse α-cardiac myosin heavy chain (mMHC) promoter24 was fused to sequences encoding ANF (G.Y. Koh, M.G. Klug, L.J. Field, unpublished data; see Figure, B). In the adult mouse, αMHC is expressed at high levels throughout the heart. Our rationale is that fusion gene expression in the ventricle should result in constitutive ANF processing in transgenic animals. Thus, elevated levels of processed ANF will circulate in mice carrying the mMHC-ANF transgene, compared with the preponderance of pro-ANF observed in the TTR-ANF mice. Additionally, expression of the mMHC-ANF transgene should be modulated pharmacologically by agents that increase or decrease expression of the endogenous αMHC gene (as, for example, thyroid hormones or propylthiouracil).35,37 Initial studies with the mMHC-ANF mice indicate that these animals also have a hypertensive phenotype. Assessment of other physiological parameters, as well as the molecular form of the immunoreactive ANF in these animals, is currently being pursued.

TTR-NTF Mice

Several reports have appeared suggesting that the amino terminal fragments (NTFs) of pro-ANF have hormonal activities.38,39 Most of these experiments used synthetic peptides based on potential protease sites in ANF.40 Administration of such NTF molecules induced vasorelaxation in vitro and hypotension in vivo. In addition, increased cGMP production was observed in isolated vessels treated with either NTFs or ANF. In the kidneys, NTF administration affected diuresis, natriuresis, and kaliuresis, as well as increased sodium transport by the Na+,K+-ATPase.43 Competitive binding studies using smooth muscle cell membranes have shown specific and separate binding of NTFs with an affinity comparable to that observed for ANF99,126 and the GC-A receptors. Of potential clinical significance, plasma NTF levels were elevated in patients with chronic renal failure and congestive heart failure.41

With respect to our TTR-ANF mice, the potential biocactivity of NTFs is important, given the low level of prohormone processing observed in that model. To assess directly the potential biologic role of NTF, we are generating mice with an altered TTR-NTF transgene, designated as TTR-NTF (Figure, C). We have engineered an in-frame stop codon that terminates propro-ANF translation at the prohormone processing site. Consequently, hepatocytes of these transgenic mice will secrete only the amino terminal portion of pro-ANF. This model, in combination with the TTR-ANF and mMHC-ANF mice, will enable us to make correlates between the biologic activities of the NTFs, unprocessed ANF, and processed ANF.

VSMA-ANFR Mice

Recent mutational analyses of the GC-A ANF receptor have shown that deletions of the protein kinase domain result in a constitutively active cyclase domain.44 We are currently exploiting this finding to generate a transdominant, constitutively active ANF receptor. Expression of this receptor then will be selectively targeted to individual ANF-responsive cell types in transgenic mice. This approach should permit independent examination of the contribution of each ANF target tissue to the hypotensive response. Our initial model uses the vascular smooth muscle actin (VSMA) promoter23 to target expression of the activated GC-A ANF receptor (ANFR) to the vasculature (Figure, D). With the use of this approach, it may be possible to systematically dissect the physiological response to ANF by specific target tissue activation.

Conclusion

Transgenic animal technology provides an experimental model system that contributes new insights to the role of ANF in long-term cardiovascular and renal regulation. During the process of defining the etiologic basis for the TTR-ANF hypotensive phenotype, we are exploring additional transgenic models. The mMHC-ANF and TTR-NTF transgenes are designed to assess the role of processed ANF and NTFs, respectively. Additionally, the VSMA-ANFR transgene, intended to mimic the cellular action of ANF on vascular smooth muscle, should further dissect the hypotensive response to chronically elevated ANF.

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