Atrial Natriuretic Factor and Transgenic Mice

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Atrial natriuretic factor (ANF) is a peptide hormone secreted by the cardiac atrium in response to myocardial stretch. Short-term administration of ANF elicits numerous physiological responses, including a prompt and pronounced natriuresis, alterations in intrarenal hemodynamics and tubular function, inhibition of the renin-angiotensin-aldosterone system, and a transient decrease in blood pressure. The various systemic actions of ANF are mediated through the guanylate cyclase (GC)-A ANF and GC-B ANF receptors, which generate intracellular cyclic GMP (cGMP) in a ligand-dependent manner. Although these short-term effects of ANF are well characterized, the role of the hormone in long-term cardiovascular regulation has remained controversial. Accordingly, we have generated a transgenic mouse model that exhibits chronically elevated plasma ANF levels. These mice carry a transgene that targets ANF mRNA expression to the liver. Hepatic ANF expression results in a 10-fold increase in plasma hormone levels compared with nontransgenic littermates. The transgenic mice exhibit a long-term hypotensive phenotype, with blood pressures typically 20 to 30 mm Hg below those observed in nontransgenic siblings. However, the expected natriuretic and diuretic effects of ANF are not observed. Thus, transgenic mice with chronically elevated plasma ANF levels appear to be able to compensate for the renal effects of hormone overexpression. Current experiments are aimed at defining the etiology for the hypotension observed in this model.

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 preprohormone. Cleavage of a 25-amino acid signal peptide generates pro-ANF (also known as ANF$_{126}$). 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 ANF$_{126}$ 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.\(^3\) 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 vasoconstriction 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.\(^3\) 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.\(^11\) 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 cANF\(_{43,12}\).

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 (\(t\_{1/2}=20\) to 180 seconds). Two major degradative pathways are responsible for the rapid clearance of ANF.\(^2\) 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.\(^11\) This suggestion is supported by the observation that administration of agonist, cANF\(_{43}\), 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.\(^17\) Long-term infusions of ANF have been observed to decrease mean arterial pressure in both normotensive\(^18\) and hypertensive\(^17\) 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 normotensive\(^17\) or hypertensive\(^19\) 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 littermates. 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 acetyltransferase (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 hypertension 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 hypertension 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 characteristic 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 vivo. These effects were independent of the ANF-induced decrease in mean arterial pressure. Recently, Lanese et al. have measured direct lumen diameter on in vitro-prepared afferent and efferent arterioles from rat kidney. ANF was shown to dilate preconstricted afferent arterioles and constrict efferent arterioles. These activities of ANF may contribute to the maintenance of normal GFR in the presence of the hypotensive phenotype. It is also of interest to note that the compensatory humoral systems are not activated by ANF-induced chronic hypotension. For example, plasma renin activity and noradrenaline and epinephrine levels are not markedly altered in the TTR-ANF mice. Similarly, vasopressin, corticotropin, and renal renin mRNA levels are not perturbed (M.E. Steinhelper, L.I. Field, unpublished observations). Aldosterone levels are elevated in the TTR-ANF mice compared with nontransgenic controls (1.37±0.21 versus 0.74±0.07 ng/mL, respectively). This latter result is directly opposed to what is observed after short-term ANF administration. Additional ongoing studies on renal function from the Sonnenberg laboratory will undoubtedly shed additional light on the mechanism of renal compensation in the TTR-ANF mice.

In collaboration with Nick Hill’s group at the Rhode Island Hospital, experiments were initiated to identify any ameliorating effects of ANF on experimentally induced pulmonary hypertension. Analyses of animals maintained in a hypoxic (0.5 atm) environment for a period of 3 weeks revealed a blunted response in both the degree of right ventricular hypertrophy and absolute increase in right ventricular pressure in transgenic mice compared with nontransgenic siblings. These results suggest that long-term elevation of plasma ANF levels can exert a cardioprotective effect on the myocardium during right ventricular hypertrophy. Recent studies by Jin and coworkers detected a similar protective effect after short-term ANF administration. Several other interesting observations were made during the chronic hypoxia study. First, Northern blot analyses revealed that cardiac ANF mRNA was not subject to feedback transcriptional regulation: both right atrial and right ventricular ANF levels were comparable between the TTR-ANF mice and nontransgenic controls. In addition, the hearts of mice maintained in a normoxic environment were found to be approximately 30% smaller in the transgenic animals compared with nontransgenic siblings, despite similar body weights between the two groups. It is possible that the observed reduction in myocardial mass is due to decreased afterload in the hypotensive transgenic animals. Alternatively, ANF may exert a direct effect on myocardial growth.

In summary, long-term elevation of plasma ANF levels in the TTR-ANF transgenic mice produced a marked hypotensive phenotype without the expected alterations in natriuresis and diuresis. Thus, it would appear that the transgenic mice are able to counteract the natriuretic effects of ANF but not the hemodynamic effects. An additional review of the physiological perturbations observed in the TTR-ANF mice has recently been compiled. Although we have not exhaustively evaluated cardiac output, total peripheral resistance, structural changes of the vascular wall, or intravascular volume in detail, several of these studies are currently in progress. Rigorous assessment of cardiovascular, renal, and hormonal function in the TTR-ANF mice is warranted to determine the precise etiology of the hypotensive phenotype.

**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 initial 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. 36 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 the 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. 38-42 Most of these experiments used synthetic peptides based on potential protease sites in ANF. 43 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. 44 Competitive binding studies using smooth muscle cell membranes have shown specific and separate binding of NTFs with an affinity comparable to that observed for ANF 99,126 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-NTF mice, the potential bioactivity 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 prepro-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 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 physiologic 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 producing 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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