Lung Is an Important Source of Atrial Natriuretic Factor in Experimental Cardiomyopathy

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Abstract

The regulation of water and electrolyte homeostasis is multifactorial and includes the heart and kidneys as important regulatory centers. Within the heart, a recently discovered hormone, atrial natriuretic factor (ANF), has been implicated in the maintenance of water and salt balance. Primarily found in mammalian atria, ANF has been detected in low amounts in several tissues, including lungs. A disorder of the ANF system has been demonstrated in genetically cardiomyopathic hamsters, a model for human congestive cardiomyopathy. Atrial ANF gene expression and storage are decreased during development of this disease, while paradoxically, circulating levels of ANF are increased. We have hypothesized that an extra-cardiac source may contribute to ANF production in these pathological conditions. In this paper we provide evidence that ANF synthesis is stimulated in the lungs of hamsters during development of cardiomyopathy as revealed by increased ANF mRNA and peptide levels. Furthermore, we show that ANF synthesized in lungs is secreted and has identical chromatographic and biological properties to circulating ANF. The increased production of ANF in lungs may be physiologically important in preventing pulmonary edema. Alternatively, during cardiac dysfunction, lungs may play a compensatory role by increasing their contribution to plasma ANF levels.

Introduction

The regulation of water and electrolyte homeostasis is under multifactorial control; some factors are responsible for the behavioral patterns of water and salt intake, while others are involved in the regulation of fluid and electrolyte retention of excretion (1). Many organs, including the heart, contribute to the maintenance of water and salt balance. Within the heart, atrial natriuretic factor (ANF),¹ a 28 amino acid peptide hormone that is released from atria after activation of stretch receptors, and perhaps other hormonal signals, appears to play an important role in the regulation of water and electrolyte

balance in concert with other hormonal systems such as renin, angiotensin, aldosterone, and vasopressin (2, 3). First isolated from mammalian atria, ANF has also been found, albeit at much lower levels, in other tissues including ventricles, brain, pituitary, adrenals, and lungs (4). The presence of ANF prohormone (5) and mRNA (6) in normal rat lungs, together with our observation that rat lungs secrete biologically active ANF (5), suggest that lungs may be a physiologically relevant source of circulating ANF under certain conditions. We tested this hypothesis using genetically cardiomyopathic hamsters, a model of human congestive heart failure and a paradigm of hereditary dystrophy-like cardiopathy and myopathy (7). Cardiomyopathic hamsters have been shown to be deficient in atrial ANF bioactivity (8) with a corresponding reduction in ANF mRNA levels (9), suggesting that ANF gene expression is deficient in the atria of these animals. Paradoxically, plasma ANF levels are markedly increased (10). These data cannot be reconciled solely by an increased secretion leading to a subsequent reduction in storage in the atria (11). These observations suggest that an extra atrial source of ANF may develop during congestive heart failure. One such source appears to be the ventricular myocardium (12). In this study we show that the lung may be another, and particularly important, source.

Methods

Animals. The cardiomyopathic hamsters (TO.2) used in this study were originally derived from BIO 53.58 cardiomyopathic hamsters (13) and have been inbred for over 20 generations. The animals were housed at room temperature in a 12-h light-dark cycle and fed normal chow. They drank water ad lib.

Three major groups of hamsters were studied. Young animals (106 d old) exhibited the myocardial lesions characteristic of cardiomyopathy, but had minimal hypertrophy and no evidence of congestion. Animals 163 and 217 d old were characterized by increasing ventricular hypertrophy and occasional mild hepatic congestion. Animals 252 and 272 d old had advanced cardiomyopathy, with cardiac dilatation, pulmonary and hepatic congestion, and subcutaneous edema. Random bred Syrian golden hamsters were used as a control.

Preparation of tissue homogenates. The animals were killed by decapitation. Lungs from each animal were immediately dissected and after washing in saline they were collected in 5 ml cold 0.1 M acetic acid containing protease inhibitors to a final concentration of 1 mg EDTA/ml, 5 μ M pepstatin A (No. P4265; Sigma Chemical Co., St. Louis, MO), and 10 μ M PMSF (No. P7626; Sigma Chemical Co.). The tissue was homogenized at 4°C for 30 s in a Polytron homogenizer. After 20 min centrifugation at 32,000 g the pellet was washed and rehomogenized in 2.5 acetic acid containing inhibitors. Both supernatants were collected together. Right and left atria were also quickly dissected and separately collected in 2 ml 0.1 M acetic acid with inhibitors. The same procedure as described for lungs was followed. The ANF immunoreactivity was determined by direct RIA as previously described (14).

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1. *Abbreviations used in this paper:* ANF, atrial natriuretic factor; RP, reverse phase; TFA, trifluoroacetic acid.

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Extraction of ANF from plasma and tissues. After decapitation, trunk blood was collected in chilled tubes containing 1 mg EDTA, 10 μ l pepstatin A 0.5×10^{-3} M, and 10 μ l PMSF 10^{-3} M/ml of blood and centrifuged at 3,500 rpm for 20 min at 4°C. The plasma was assayed immediately or stored at -70°C. The ANF was extracted from plasma by heat-activated glass beads (Vycor; Corning Glass Works, Corning, NY) (15). To 1 ml of hamster plasma, 0.5 ml of activated glass suspension (Vycor; 50 mg in 1 ml distilled water) was added and agitated slowly in a cold room (4°C) for 30 min. After 3 min centrifugation at 3,000 rpm the supernatant was aspirated and the glass powder was washed with 1.5 ml deionized water. The adsorbed ANF was eluted from the glass powder with 1 ml of 60% acetone in 0.05 M HCl. The acetone was evaporated in a nitrogen stream and the aqueous phase was lyophilized in a Speed-Vac. Just before the assay the residue was dissolved in RIA buffer.

For further purification on reverse phase (RP)-HPLC, tissue ANF was extracted from homogenates on Sep-Pak cartridges that were activated by 8–10 ml acetonitrile and then washed with 8–10 ml ammonium acetate, pH 4.0. The adsorbed ANF was eluted with 3 ml 60% acetonitrile in ammonium acetate (0.2%, pH 4.0). The organic solvent was evaporated under a nitrogen stream followed by lyophilization in a Speed-Vac (16).

RIA for ANF. ANF immunoreactivity was determined by a double antibody RIA (14). The sensitivity of this assay was ~ 0.75 pg.

Reverse phase HPLC. The lyophilized tissue extracts were dissolved in 10% acetonitrile and 0.1% trifluoroacetic acid (TFA) and injected on a C₁₈ μ m Bondapak column (3.9 mm $\times$ 30 cm) in a (LKB Instruments, Inc., Gaithersburg, MD) HPLC system. The elution of ANF from the column was performed with a linear gradient of 15–50% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The 2-ml fractions were collected. ANF immunoreactivity was determined by direct RIA (14).

RNA analysis. Animals were killed by decapitation and lungs, ventricles, and atria were dissected and quickly frozen in liquid nitrogen. Measurements were performed on two groups, each consisting of 10 controls and 5 cardiomyopathic animals. Total RNA was extracted and processed for Northern blot hybridization as previously described (17) using a ³²P-labeled 600-bp Pst I fragment of rat ANF cDNA (18). The amount of total RNA was quantitated using ultraviolet spectroscopy, and the integrity of the RNA was checked by agarose gel electrophoresis. The amount of total mRNA in each RNA sample was evaluated by dot blot hybridization to a ³²P-labeled oligo(dT)_{12–18} probe. Finally, a rat brain β -actin cDNA probe (19) was used as internal

control in rehybridization experiments. The amount of specific mRNA species was quantitated by densitometric scanning of the autoradiographs. No significant change in the level of β and γ actin mRNAs was observed among the different groups.

Hamster lung perfusion. Hamsters were anesthetized with an intraperitoneal injection of pentobarbital (1.6 mg/100 g body wt) after they were killed, and the lungs perfused according to the technique of Engineer et al. (20) as we have previously described. Briefly, the heart and lungs were totally removed and rapidly perfused with oxygenated Krebs buffer via pulmonary artery. The lungs were perfused initially at a rate of 10 ml/min and inflated several times to eliminate all traces of blood. After the cannula inside the pulmonary artery was tied, the heart was discarded and the lungs were then perfused at a rate of 2 ml/min. Collection of the effluent was started after a 5-min stabilization period. The perfusates were collected with an equal vol of 0.2 M acetic acid containing the protease inhibitors to give a final concentration of 1 mg EDTA/ml, 10 μ M PMSF, and 5 μ M pepstatin A. The ANF immunoreactivity was measured in the perfusate by RIA after prior extraction on Sep-Pak cartridges.

Radioreceptor assay. The biological activity of ANF isolated from hamster lung perfusate was assessed by radioreceptor assay using rat kidney glomerular membranes (21). Rat glomerular membranes were prepared in 0.05 M Tris-HCl buffer, pH 7.4, containing 1 μ M aprotinin (No. 817113; Miles Laboratories, Inc., Elkhart, IN), 0.1% bacitracin (No. 80125; Sigma Chemical Co.), 500 μ M PMSF, 5 mM MgCl₂, and BSA (lot 405; Miles Laboratories, Inc.). Synthetic (Arg 101-Tyr 126) ANF was labeled with I¹²⁵ by the lactoperoxidase method and the tracer was purified by RP-HPLC. Specific activity of the tracer was ≈ 950 μ Ci/ μ g. Membrane proteins (35 μ g in 50 μ l) were incubated with $\approx 10,000$ cpm ¹²⁵I-ANF in the presence of cold ANF (6–6250 pg/ml) or serial dilution of hamster lung perfusate HPLC fraction for 1 h at room temperature in a total vol of 1 ml.

The membrane-bound tracer was separated by filtration on GF/C paper (Whatman Laboratory Products Inc., Clifton, NJ). The filters were washed twice with 3 ml 0.05 M Tris-HCl, pH 7.4, and counted in a gamma counter (LKB Instruments, Inc.).

Results

ANF content in atria, lung, and plasma of cardiomyopathic and control hamsters. Pulmonary and atrial ANF were determined in homogenates by direct RIA (14). Dilution curves of

Table I. ANF Content in Plasma, Lungs, and Atria of Manual and Cardiomyopathic Hamsters

	Age	n	Plasma ANF (pg/ml)	Lung ANF ng/hamster (ng/mg protein)	Atrial ANF μ g/hamster (μ g/mg protein)	
					Right	Left
<i>d</i>						
Control	240	8	25.7 $\pm$ 8.5	16.1 $\pm$ 1.5 (0.44 $\pm$ 0.04)	1.78 $\pm$ 0.12 (0.90 $\pm$ 0.05)	1.04 $\pm$ 0.04 (0.54 $\pm$ 0.03)
Control	270	8	22.3 $\pm$ 8.4*			
Cardiomyopathy	106	8	85.8 $\pm$ 14.9**			
Cardiomyopathy	163	8	150.1 $\pm$ 30.9†	35.1 $\pm$ 8.3† (0.96 $\pm$ 0.25)	1.36 $\pm$ 0.04† (0.64 $\pm$ 0.03)	0.76 $\pm$ 0.06† (0.40 $\pm$ 0.14)
Cardiomyopathy	217	6	238.3 $\pm$ 31.7**			
Cardiomyopathy	252	6	269.2 $\pm$ 52.8†	64.7 $\pm$ 10.6† (2.1 $\pm$ 0.04)	0.98 $\pm$ 0.14† (0.40 $\pm$ 0.07)	0.68 $\pm$ 0.04† (0.37 $\pm$ 0.11)
Cardiomyopathy	272	6	310.0 $\pm$ 29.7**			

Values are expressed as mean $\pm$ SE. Please note the differences in units between lung (nanograms/milligram protein) and atrial (micrograms/milligram protein) ANF values. * The lungs and atria of these animals were processed for measurement of ANF mRNA. † $P < 0.001$ as compared with control animals.

pulmonary and atrial homogenates of control and cardiomyopathic hamsters were parallel to the RIA standard curve, indicating that the homogenates contain a peptide indistinguishable from synthetic ANF. As shown in Table I, the total atrial content of ANF was significantly lower in the animals with moderate cardiomyopathy, and decreased further in animals with severe heart failure. The concentration of ANF as expressed in micrograms per milligram protein was also lower, while the plasma content increased with the development of the disease to ~ 14-fold higher in severe heart failure. In contrast to atrial ANF, total lung ANF content was increased twofold in the moderate state of the disease and fourfold in heart failure.

Molecular forms of ANF in plasma and tissues of cardiomyopathic and control hamsters. To confirm that increased lung ANF was due to increased peptide synthesis in the lung

Figure 1. RP-HPLC analysis of plasma, lung, and atrial tissue. Plasma ANF was extracted by heat-activated Vycor glass beads and lung and atrial tissue homogenates were extracted on Sep-Pak cartridges. The plasma and tissue extracts were applied on C₁₈- μ m Bondapak column (3.9 mm $\times$ 30 cm) in a HPLC system (LKB Instruments, Inc.). The column was developed at a flow rate of 1 ml/min using an acetonitrile gradient 15–50% in 0.1% TFA. The elution profile was monitored at 215 nm (not shown). The abscissa is marked at 10-min intervals. The arrow indicates the elution time of purified rat 126 amino acid prohormone (ANF 1–126). The 100- μ l eluates were evaporated to dryness in Speed-Vac and ANF was determined directly by RIA.

Figure 2. Northern blot analysis of ANF mRNA in atria, ventricles, and lung of control (C) and cardiomyopathic (CM) hamsters. The amount of total RNA is indicated for each lane. Total RNA was quantitated using ultraviolet spectroscopy and the amount of mRNA was quantitated by hybridization to oligo(dT)_{12–18} as described in Methods. The amount of RNA in the four lung samples was identical as further assessed by rehybridization of the same blot with a β -actin probe as detailed in Methods.

and was not the result of increased uptake of circulating ANF by high affinity binding sites in lung epithelium (22), we analyzed the molecular weight forms of lung ANF. The HPLC elution pattern of plasma, atrial, and pulmonary extracts is shown in Fig. 1. Multiple forms of ANF in cardiomyopathic hamster lungs are observed. Two of the peaks were identified, by comparison of elution time, as the 126 amino acid prohormone (Asn 1-Tyr 126) and the 28 amino acid circulating peptide (Ser 99-Tyr 126). The other peaks may be degradation products, although their identity is unknown at this time.

Tissue ANF mRNA. To determine if increased lung ANF was due to enhanced ANF gene expression, we measured ANF mRNA in lung tissue and compared it with atrial ANF mRNA content. As shown in Fig. 2, ANF mRNA in lung is not detectably changed in cardiomyopathic hamsters at the onset of development of hypertrophy (106 d old). With progression of the disease, ANF mRNA increased in the lung; in cardiomyopathic animals who have developed cardiac hypertrophy but are not in congestive heart failure, pulmonary ANF mRNA increased twofold. Lung ANF mRNA was greatest (fivefold increase over control) in animals (272 d old) who were in congestive heart failure. In contrast, atrial ANF mRNA levels are slightly decreased (40%) in the same cardiomyopathic animals as compared with the controls, while ventricular ANF mRNA is significantly increased as previously reported (9).

Hamster lung perfusion. To examine the secretion of ANF from lungs, the perfusion of this organ was performed. As shown in Fig. 3 A, ANF was released in a time-dependent manner during a 60-min perfusion of cardiomyopathic and normal hamster lungs. The HPLC pattern of cardiomyopathic hamster lung perfusates was identical to the pattern of plasma ANF (Fig. 3 B). We further tested the biological activity of lung-produced ANF by its ability to displace ¹²⁵I-ANF from rat glomerular receptors (21). The ANF purified from cardiomyopathic hamster lung perfusates did indeed compete in a dose-dependent manner with ¹²⁵I-ANF for rat glomerular receptors (Fig. 4), suggesting that it possesses the potential for full biological activity.

Figure 3. (A) Effect of perfusion time on ANF release by representative hamster lungs. 1.5 ml of each 2-ml fraction of lung effluent was extracted by Sep-Pak cartridges and ANF was measured in the extracts by RIA. ●, cardiomyopathic hamsters; ○, control. (B) Representative RP-HPLC performed on pool of effluent of cardiomyopathic hamster lungs. The C_{18} - μ m Bondapak column (3.9 mm $\times$ 30 cm) was eluted with the 15–50% acetonitrile gradient in 0.1% TFA. The immunoreactive ANF was determined in 100 μ l of each fraction directly by RIA.

Discussion

The results presented in this paper clearly show that lungs synthesize an increased amount of authentic ANF in a model of genetic cardiomyopathy, particularly during the development of congestive heart failure. During the development of disease, atrial ANF synthesis is diminished while plasma ANF is increased. This is accompanied by an increased content of lung ANF. Our study of the molecular forms of ANF present in the lungs shows that the main form is a 126 amino acid prohormone (Asn 1-Tyr 126). Since the circulating peptide is mainly a 28 amino acid fragment, the presence of a large proportion of prohormone in lungs rules out the possibility that increased pulmonary ANF content is simply due to increased

uptake from the circulation. Furthermore, we tested directly whether increased peptide content reflects increased ANF gene expression by measuring mRNA in atria and lungs of control and cardiomyopathic hamsters. Changes in mRNA levels closely paralleled changes in peptide concentrations. Increases in lung ANF mRNA were detected after the onset of cardiomyopathy and were greatest in congestive heart failure. Before cardiac decompensation, at 106 d of age, there was little change in ANF mRNA levels in the lung. With development of cardiomyopathy, ANF peptide content (examined at 163 d of age) and ANF mRNA levels (at 217 d) were increased two-fold. When the animals were in congestive heart failure (252 and 272 d of age) both ANF mRNA and peptides were increased four- to fivefold. These data indicate that the expression of the ANF gene in the lung of genetically cardiomyopathic hamsters is not altered until development of hemodynamic changes that accompany the cardiomyopathic process.

In the lung of animals with congestive heart failure, ANF mRNA and peptide concentrations are 100-fold lower than in atria; however, since the mass of lung tissues is at least 50-fold greater than atrial tissues, the lungs may produce amounts of ANF similar to those produced in atria and thus contribute to the elevated levels of plasma ANF in these animals. The observation that authentic ANF can be secreted from lungs strengthens this hypothesis. It is important to mention that ANF gene expression is increased also in the ventricles of cardiomyopathic hamsters (12). However, the exact contribution of ventricles to plasma ANF remains to be established and the physiological significance of increased ANF mRNA in diseased ventricles is being investigated.

The differential control of ANF gene expression in atria and extraatrial tissues, particularly in lungs, is intriguing and may be attributed to a differential response of the two tissues to changes in hormonal and neuronal activities. Indeed, the early phase of congestive heart failure is associated with increased sympathetic activity (23) and a reduced capacity to excrete water or salt (24) that may be due to impairment of cardiac ANF secretion. The increase in pulmonary ANF production may be a compensatory response to sympathetic stimulation and to the decrease in atrial ANF secretion. This response may play a protective role in preventing further increases in peripheral vascular resistance by increasing the level of plasma ANF.

Alternatively, stimulated synthesis of ANF in the lungs may have a physiologically important paracrine function in preventing pulmonary edema that develops in conditions of congestive heart shock, renal disease, and lung infections. In support of this hypothesis, two studies have recently shown that administration of ANF can prevent experimentally induced pulmonary edema (25, 26). Thus, during cardiac dysfunction, extraatrial tissues like lungs may play an integral part in regulating body fluid volume and electrolyte balance by increasing their production and secretion of biologically active ANF. This is a first observation of metabolic changes in the lungs in experimental cardiomyopathy.

Figure 4. Representative standard curve for radioreceptor assay using rat kidney glomerular membranes expressed as logit B/Bo vs. ANF concentration. Bo is the radioactivity bound to the membrane receptors. B is the radioactivity bound to the receptors in the presence of known quantity of ANF standards. △, Serial dilution (1, 4, 8, 16 μ l) of HPLC fraction (eluted with 33% acetonitrile) from cardiomyopathic hamster lung perfusates.

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