The N-Terminus of the Atrial Natriuretic Factor Prohormone in the Pleural Fluid of Congestive Heart Failure Patients*

David L. Vesely, M.D., Ph.D.; Chris J. Winters, M.D.; and Alan L. Sallman, M.D.

To investigate the possibility that the prohormone of atrial natriuretic factor might be secreted into the pleural fluid of patients with congestive heart failure who are known to have high concentrations of both the N-terminus and C-terminus of this prohormone circulating in their plasma, six patients with class 2 New York Heart Association classified congestive heart failure had the simultaneous measurement of plasma and pleural fluid N-terminal and C-terminal atrial natriuretic factor prohormone concentrations. The 98 amino acid (aa) N-terminus, the midportion of the N-terminus consisting of aa 31-67 of the 126 aa ANF prohormone (ie, pro ANF 31-67), and the C-terminus (aa 99-126, ANF) were found in high concentrations in the pleural fluid of all of these patients. The concentrations of the N-terminus (ie, pro ANF 1-98), and pro ANF 31-67 in pleural fluid were nearly equal to their concentration in plasma of these patients. Their plasma levels were more than double the plasma concentrations of pro ANFs 1-98 and 31-67 in 54 persons without congestive heart failure. These preliminary findings demonstrate that all 126 amino acids of the ANF prohormone are present in pleural fluid of patients with congestive heart failure since both the 98 aa N-terminus and the C-terminus (aa 99-126) are present. Whether or not the N-terminus, which contains diuretic and natriuretic peptides, secretion into pleural fluid helps clear the fluid present in the lung in congestive heart failure could not be determined from the present investigation.

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Atrial natriuretic factor, a new peptide hormone with potent natriuretic and vasodilatory properties that was originally characterized from cardiac atrial tissue by Debold et al., is increased in the plasma of patients with congestive heart failure. Two peptides consisting of amino acids 1-30 and 31-67 of the amino-(N)-terminal end of the 126 aa prohormone of atrial natriuretic factor have also been found to have vasodilatory and natriuretic properties. The N-terminus of the prohormone, as well as the carboxy (C)-terminus (ie, ANF consisting of aa 99-126) circulates normally in humans. The circulating concentrations of the N-terminus and the C-terminus in persons with congestive heart failure increase proportionately to the severity of the abnormality of salt and water metabolism. Recently, atrial natriuretic factor has been demonstrated to be present in the pleural fluid of congestive heart failure patients. The present investigation was designed to determine if patients with congestive heart failure secrete the N-terminus, containing the natriuretic peptides pro ANF 1-30 and pro ANF 31-67, into their pleural fluid in an attempt to rid their lungs of the excess fluid that is present in this condition. In this preliminary investigation, the whole N-terminus (ie, aa 1-98), the midportion of the N-terminus consistent with aa 31-67, and the C-terminus (ie, ANF) were found in the pleural fluid of all patients with congestive heart failure. Whether or not these newly described peptide hormones in the pleural fluid help the lung clear the fluid present in congestive heart failure could not be determined from the present investigation.

Patients and Methods

Subjects

Six volunteers with class 2 New York Heart Association classified congestive heart failure had the concentration of the whole N-terminus (ie, aa 1-98), the midportion of the N-terminus (ie, aa 31-67) and the C-terminus (ie, aa 99-126, ANF) of the 126 aa ANF prohormone measured in pleural fluid obtained from a diagnostic thoracentesis by our previously described methods. A simultaneous plasma sample from the brachial vein was obtained for comparison of the concentration of the N-terminus in plasma and pleural fluid. These six patients had respiration of 12 to 18 per minute with heart rates of 76 to 92 beats per minute. Their blood pressures ranged from 110/68 to 138/88. None of these congestive heart failure patients had any discernible renal impairment with the serum creatinine values of all subjects being below 1.5 mg/dl. The size of the pleural effusions in these patients varied from one eighth to one half of one side of the chest based upon physical examination and confirmed by chest x-ray film. Four of these
patients were receiving digoxin for therapy at the time of their thoracentesis but diuretics were discontinued in these patients for at least 24 hours prior to thoracentesis. The other two subjects presented to the hospital with symptoms of congestive heart failure for the first time. These subjects were receiving no medications, and their thoracentesis was performed before beginning any therapy. Informed consent was obtained from each of these congestive heart failure patients after the nature and possible consequences of the study were fully explained. This investigation was approved by the Human Use Committees of both the University of Arkansas for Medical Sciences and the John L. McClellan Memorial Veterans Hospital.

**Plural Fluids**

The mean protein concentration of the six thoracentesis samples was 0.6 g/L with no individual protein concentration above 0.5 g/L in the respective plural fluids. All of these fluids were thus, transudates which were handled as follows: 10 ml of each transudate was centrifuged at 3,000 g for 15 minutes and then 100 µl of the supernatant was extracted with 100 percent ethanol (1:1 dilution at 4°C), vortexed, and allowed to stand for 15 minutes. After this 15-minute period, the plural fluids were re-centrifuged at 3,000 g for 15 minutes and the supernatants taken to dryness with controlled nitrogen flow. The plasma samples (100 µl of each) were extracted the same as the plural fluids. Both plasma and plural fluids were then examined for their concentration of pro ANF 1-98 (the whole N-terminus of prohormone), pro ANF 31-67 (midportion of N-terminus), and C-terminus (ANF).

**Radioimmunoassays for Pro 1-98, Pro ANF 31-67 and ANF**

Radioimmunoassays to measure the N-terminus of the prohormone were devised to aa 1-30 and 31-67 of the 126 aa prohormone while the C-terminal assay measures amino acids 99-126 of the prohormone, i.e., ANF, as previously described by our laboratory.16-18 Our pro ANF 1-30 radioimmunoassay recognizes a component in plasma of approximately 10,000 mw as characterized by gel-permeation chromatography (G-50 Sephadex) which is consistent with the whole N-terminus of the ANF prohormone (i.e., aa 1-98), but without the C-terminus attached to it.19 The pro ANF 31-67 radioimmunoassay recognizes in plasma a component of the N-terminus of approximately 9600 mw which is consistent with measuring only aa 31-67 of the prohormone (i.e., pro ANF 31-67, mw 3878). Our ANF radioimmunoassay recognizes a 3,000 mw peptide in plasma with ANF's actual molecular weight being 3,081. All determinations were performed in triplicate. The interassay coefficient of variation for pro ANFs 1-30, 31-67, and ANF radioimmunoassays were 4.8 percent, 5.3 percent, and 5.7 percent, respectively. The interassay coefficient of variation was 8 percent for both pro ANFs 1-30 and 31-67 radioimmunoassays while ANF's radioimmunoassay interassay variation was 6.9 percent.

Recovery was examined by adding synthetic unlabeled pro ANF 1-30, pro ANF 31-67 and ANF at 100, 200, and 400 pg/ml to pooled plasma. Recovery for pro ANF 1-30 was 83.5 ± 13.2 (SD) percent while pro ANF 31-67 recovery was 100.9 ± 8.9 percent. Recovery of ANF was 92 ± 11 percent. The respective IC50s were 180, 120 and 11 fmol/tube while the lowest detectable concentrations were 40, 35 and 1.4 fmol for pro ANFs 1-30, 31-67 and ANF radioimmunoassays, respectively. Serial dilution of pooled plasma has revealed excellent parallelism of standard and unknown in these assays.16,18 Reverse phase high pressure liquid chromatography utilizing 5 micron cartridge columns revealed that the pro ANFs and ANF measured were authentic.

**RESULTS**

The concentrations of immunoreactive pro ANF 1-98 (whole N-terminus) and ir pro ANF 31-67 (midportion of the N-terminus) in the pleural fluid of each of six individuals with New York Heart Association (NYHA) class 2 congestive heart failure are shown in Figure 1. The mean concentration of pro ANF 1-98 in the pleural fluid of these patients was 1631 ± 147 fmol/ml (SEM). The average concentration of pro ANF 31-67 in their pleural fluids was 1,690 ± 199 fmol/ml. The respective circulating concentrations of the whole N-terminus and pro ANF 31-67 in each of these same individuals is also illustrated in Figure 1. It is important to note that the concentrations of the whole N-terminus of the ANF prohormone and pro ANF 31-67 in pleural fluid were almost as high as their concentrations in plasma. This finding is similar to ANF (C-terminus of prohormone) whose concentration in pleural fluid and in the circulation was found to be nearly equal in these same patients.13

The mean concentrations of pro ANF 1-98 and pro ANF 31-67 in the circulation of these patients with class 2 NYHA congestive heart failure were 1,682 ± 146 fmol/ml and 1,832 ± 227 fmol/ml, respectively. These plasma concentrations are significantly higher (p<0.001) than that of persons without congestive heart failure, with our normal range for the whole N-terminus of the prohormone being 506 to 556 fmol/

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**Figure 1.** Comparison of the concentration of pro ANF 1-98 (the N-terminus of the ANF prohormone) and pro ANF 31-67 in the pleural fluid and plasma of six congestive heart failure patients. The normal range for pro ANF 1-98 (closed circles) and pro ANF 31-67 (closed squares) in plasma of 54 persons without congestive heart failure is indicated by the respective boxes in this figure. The concentration of both pro ANF 1-98 and pro ANF 31-67 in the pleural fluid and plasma of congestive heart failure patients was significantly higher (p<0.001) than that in the plasma of persons without congestive heart failure when analyzed by the Student's t-test for unpaired values.
ml while the circulating concentration of pro ANF 31-67 in 54 persons without congestive heart failure has been found to be 371 ± 18 fmol/ml.10

**DISCUSSION**

The present investigation demonstrates that the whole N-terminus (ie, aa 1-98) of the 126 aa ANF prohormone is present in pleural fluid of patients with congestive heart failure. With the previous demonstration that the C-terminus (ie, ANF, aa 99-126) is present in pleural fluid,13 it is now evident that all 126 aa of the ANF prohormone are present in the pleural fluid of congestive heart failure patients. Whether the whole prohormone is secreted intact into the pleural fluid or if the prohormone has been proteolytically cleaved into the C-terminus and N-terminus, with further proteolytic processing to form pro ANF 31-67 prior to secretion into the pleural fluid cannot be determined with certainty from the present investigation. If secreted as an intact prohormone, it is obvious, however, from the data of the present investigation that the proteolytic processing to form ANF and pro ANF 31-67 is very rapid as the concentration of pro ANF 31-67 from the midportion of the N-terminus is very high in pleural fluid and almost equal to its concentration in plasma. The C-terminus (ANF), likewise, is present in high concentration in the pleural fluid of these patients,13 indicating that it also has been cleaved off the intact prohormone. Thus, if the whole prohormone is secreted intact into pleural fluid, it is rapidly processed proteolytically to cleave off aa 99-126 (ANF) and to form pro ANF 31-67. The formation of pro ANF 31-67 suggests that other peptides on either side of pro ANF 31-67 in the N-terminus consisting of amino acids 1-30 and aa 68 to 98 or smaller fragments thereof are also in the pleural fluid since the intact 1-98 aa N-terminus from which they would be formed was found in pleural fluid in the present investigation. The exact amino acid length of these other peptides from the N-terminus has not been determined at present.

The etiology of the N-terminus of the ANF prohormone in pleural fluid is unknown, but two possibilities are suggested by the present data. One possibility for the N-terminus of ANF prohormone being present in pleural fluid is that it is due to capillary leakage of this relatively small (10,000 mw) peptide into pleural fluid from capillaries or postcapillary venules in the lung. Under normal conditions, the rate at which water molecules diffuse through capillary membranes is approximately 80 times as great as the rate at which plasma itself flows linearly along the capillary.15 The relative permeability of 3,000 to 5,000 molecular weight peptides such as pro ANF 1-30 or ANF is 20 percent of that of water under normal conditions while a 10,000 mw peptide like the whole N-terminus of the ANF prohormone is only 1 percent as permeable as water.16 Albumin with a molecular weight of 69,000 is <0.0001 as permeable as water under normal conditions.15 With capillary leakage, medium-sized peptides such as albumin become freely permeable through capillaries,16 implying that smaller-sized peptides such as the N-terminus of the ANF prohormone should also be freely permeable as well. Although the ability of the lung to extract either the whole N-terminus or pro ANF 31-67 from the pulmonary circulation has not been investigated, the lung has been shown to remove ANF from the pulmonary circulation.17 With a single bolus injection of 125 ANF, 67 percent of ANF is removed in a single passage through rabbit lungs as measured by indicator-dilution methods.17

A second possibility is that the whole ANF prohormone is being made in the lung and secreted directly into the pleural fluid. Pleural fluid originates from the lung rather than the pleura,18 and it is possible that both the N-terminus and C-terminus of the ANF prohormone are secreted simultaneously with this fluid into the pleural space. Now that the whole prohormone (ie, all 126 amino acids) has been found in pleural fluid, this possibility is even stronger than previously when only ANF was found in the pleural fluid.13 The presence of all 126 aa of the prohormone in pleural fluid plus evidence that perialveolar cells in lung express the ANF gene19 suggests that whole prohormone for ANF may be synthesized in the lung. Sirois and Gutkowsam have recently perfused the lungs of four human fetuses in vitro with oxygenated Krebs solution for 30 minutes and found ANF in the Krebs solution that had been perfused through the lungs suggesting that the lungs are capable of secreting ANF into the perfusate. Although these authors did not investigate whether the whole ANF prohormone or the 98 aa N-terminus of the prohormone was excreted into the perfusate, one would suspect from the data of the present investigation that the whole N-terminus as well as the C-terminus is secreted into the perfusate of isolated perfused lungs. If the lungs are synthesizing and secreting a large amount of the N-terminus and C-terminus of the ANF prohormone in persons with congestive heart failure, it is possible that their nearly equal concentrations in the plasma and pleural fluid of patients with congestive heart failure is due to the lung producing a significant amount of the plasma concentration of both the N-terminus and C-terminus of the ANF prohormone, as well as their pleural fluid concentrations in this disease.

The N-terminus of the ANF prohormone contains peptides (ie, pro ANF 1-30 and pro ANF 31-67) with similar diuretic and natriuretic properties to ANF. The finding of the whole N-terminus of the prohormone and pro ANF 31-67 itself in the pleural fluid suggests the possibility that these peptides are se-
creted along with ANF into the pleural fluid to help clear the fluid present in the pleural space. ANF has been shown to have a protective effect on the production of pulmonary edema in a study where 200 ng/ml of ANF was added 3 to 5 minutes before the infusion of various chemicals which cause pulmonary edema in isolated perfused lungs. This protective effect was not secondary to the systemic natriuretic or diuretic action of ANF since this was an isolated lung preparation. Although the mechanism of the protective effect on the production of ANF was not defined in this study, it does suggest that ANF secreted locally by the lung may have a local protective mechanism to prevent the formation of fluid in the lung. A similar study with the natriuretic peptides derived from N-terminus of the ANF prohormone has not yet been done. Whether or not the secretion of these natriuretic and diuretic peptides from the N-terminus and C-terminus of the ANF prohormone into the pleural fluid helps the lung clear the fluid in the lung in CHF, in addition to these peptides known effects of increasing salt and water diuresis by their actions on the kidney, awaits further investigation. It is of interest in this regard that postcapillary venule leakage is generally caused by agents that induce smooth muscle contraction and is inhibited by agents with smooth muscle relaxant properties. Pro ANFs 1-30 and 31-67 from the N-terminus and ANF are endogenous peptides with vascular smooth muscle relaxing properties. Their presence in increased quantities in the circulation of congestive heart failure patients as found in the present investigation should help to reverse the increased permeability of capillaries and postcapillary venules if smooth muscle vascular contraction is indeed the cause of increased capillary permeability. The smooth muscle relaxing properties of these newly discovered peptides from the N-terminus and C-terminus of the ANF prohormone should help to decrease edema formation in the lung (and elsewhere in the body) if smooth muscle contraction resulting in a larger pore opening between these cells in the vasculature is the cause of the increased capillary permeability.

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