Atrial Natriuretic Factor Immunoreactivity in Human Fetal Lung Tissue and Perfusates

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SUMMARY Immunoreactive atrial natriuretic factor (ANF) was detected in human fetal homogenates and perfusates using a sensitive and specific radioimmunoassay for the 28 amino acid (C-terminal) fragment. Three peaks of ANF immunoreactive material were found in the lung homogenates. With high performance liquid chromatography, the elution characteristics of the first immunoreactive peak were the same as those of circulating human ANF. The other two peaks have not been characterized, although one had a position similar to the 126 amino acid rat prohormone (Asn 1–Ile 110–Tyr 126). The time course of release of immunoreactive ANF by perfused human fetal lungs was also studied. It is suggested that ANF may play a role in early pulmonary function.

Atrial natriuretic factor (ANF) was originally characterized in atrial tissue by de Bold et al.1 It is a group of peptides involved in regulating fluid and electrolyte balance and maintaining systemic blood pressure.1,2 More recently, the presence of this peptide was demonstrated in various endocrine and neural tissues, including the brain,3 hypothalamus,4 ganglia of the autonomic nervous system,5 and pituitary gland.6 ANF immunoreactive materials were also detected in rat lungs.7 Furthermore, Gutkowska et al.8 characterized four forms of immunoreactive ANF (ir-ANF) in rat lung homogenates, including the 126 amino acid prohormone, and showed that only a low-molecular-weight fragment (28 amino acids, C-terminal) was released during lung perfusion. These observations together with the recent data of Gardner et al.9 demonstrating the presence of ANF messenger RNA suggest that besides the heart, the lungs may be another source of ir-ANF. We demonstrated the presence of ir-ANF in human fetal lung homogenates and perfusates.

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Key Words • secretion • perfusion • fetus

Materials and Methods

Perfusion of Human Fetal Lungs

Four fetal human lungs were obtained from elective abortions of 16- to 18-week-old fetuses. Abortions were induced by injecting hypertonic saline solution in the amniotic fluid; they were complete after 12 to 18 hours. The heart and the lungs were dissected, placed in cold Krebs solution containing heparin, 10 U/ml, and transferred to the laboratory. The ductus arteriosus was tied up, the apex of the heart was cut, and a cannula was inserted in the pulmonary trunk. The heart was carefully removed and the lungs were transferred to the perfusion apparatus where they were perfused with oxygenated Krebs solution containing heparin, 10 U/ml, and transferred to the laboratory. The ductus arteriosus was tied up, the apex of the heart was cut, and a cannula was inserted in the pulmonary trunk. The heart was carefully removed and the lungs were transferred to the perfusion apparatus where they were perfused with oxygenated Krebs solution containing heparin, 10 U/ml, and transferred to the laboratory. The lungs were collected in 2 ml aliquots on ice for 30 minutes and mixed with an equal volume of 0.1 M acetic acid containing the protease inhibitors (final concentration): EDTA (1 mg/ml), phenylmethylsulfonyl fluoride (PMSF; 10⁻³ M), and pepstatin A (5 × 10⁻⁴ M). The ir-ANF in the perfusates was measured by specific radioimmunoassay after extraction with Sep-Pak cartridges (Waters Associates, Milford, MA, USA) as described.10 The recovery of ¹²⁵I-ANF was 75 to 85%.

Preparation of Human Fetal Lung Homogenates

After the perfusion, the human fetal lung tissue was collected in 20 ml of cold 0.1 M acetic acid containing protease inhibitors to the final concentration: EDTA (1 mg/ml), PMSF (10⁻³ M), and pepstatin A (5 × 10⁻⁴ M).
The tissue was homogenized twice on ice for 30 seconds with a Polytron homogenizer. After 20-minute centrifugation at 32,000 g, the supernatant was separated and kept on ice, and the pellet was rehomogenized in 10 ml of 0.1 M acetic acid with inhibitors and centrifuged again. Both supernatants were collected together and frozen at −70°C until assayed or extracted.

**Extraction of Immunoreactive ANF from Lung Tissue**

The irANF was extracted from acetic acid homogenates of human fetal lung by Sep-Pak cartridges as described previously for human plasma. Briefly, the cartridges were prepared by activating with 8 to 10 ml of acetonitrile and washing with 8 to 10 ml of 0.2% ammonium acetate, pH 4.0. The homogenates (2-4 ml) were applied on the activated Sep-Pak cartridges, which were then washed with 0.2% ammonium acetate, pH 4.0. The adsorbed irANF was eluted with 3 ml of acetonitrile (60%) in ammonium acetate (0.2%, pH 4.0). The organic solvent was evaporated under nitrogen stream after lyophilization in a Speed-Vac (Farmingdale, NY, USA). The residue was taken up in 500 µl of 0.1 M phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin and 0.1% Triton X-100 before the assay.

**Radioimmunoassay for ANF**

ANF immunoreactivity was determined by radioimmunoassay as previously reported.

**Reverse-Phase High Performance Liquid Chromatography**

The lyophilized tissue extracts were dissolved in 10% acetonitrile and 0.1% trifluoroacetic acid (TFA) and applied to a C18 μBondapak column (0.39 x 30 cm) in an LKB HPLC system (Broma, Sweden). The elution from the column was performed in 0.1% TFA with a linear gradient of 15 to 50% acetonitrile at a flow rate of 1 ml/min. The 2-minute fractions were collected. ANF immunoreactivity in the HPLC fractions was determined directly by radioimmunoassay. Synthetic human C-terminal peptide ANF-(99-126) was chromatographed under identical conditions and served as standard.

**Results**

Series of experiments were done to characterize the presence of irANF in the lung tissues and perfusates. In the initial series, an extract of fetal lung homogenates obtained as described in Materials and Methods was submitted to serial dilution and tested in our very sensitive radioimmunoassay for human ANF. As shown in Figure 1, diluted aliquots of lung extract (aliquots of 5, 10, 20, and 50 µl of lung homogenates) gave a proportional displacement of radioactive ANF (123I-ANF), which fits perfectly with the binding curve of pure human ANF-(99-126). An average of 28.2 ng (n = 3) of irANF was found in the fetal lungs.

In the second set of experiments, fetal human lung extracts were submitted to reverse-phase HPLC separation, and the elution pattern is shown in Figure 2. Three peaks containing irANF material (see Figure 2) were present in the homogenates of human fetal lungs. The elution characteristics of the first immunoreactive peak (fraction 16) were the same as those of the circulating human ANF (28 amino acid C-terminal peptide; Ser 99-Met 110-Tyr 126). The other two immunoreactive peaks have not yet been characterized. Nevertheless, the immunoreactive peak eluting with the highest acetonitrile concentration (fractions 30-32) has a similar position to the elution pattern of rat 126 amino acid prohormone (Asn 1-Ile 110-Tyr 126). Because of the scarcity of material, amino acid analysis was not performed.

Figure 3 illustrates the time course of release of irANF by perfused human fetal lungs. Aliquots of lung perfusates contained approximately 50 pg per 3 ml of perfusates at the beginning of perfusion. The content decreased during the 30-minute perfusion and became undetectable at the end of the experiment.

**Discussion**

These results show that extracts of human fetal lungs contain several forms of human irANF. We have presented evidence showing that the most abundant form was the 28 amino acid fragment (C-terminal peptide). The immunoreactivity of this fragment was similar to the synthetic 28 amino acid human ANF in the radioimmunoassay. The presence of human irANF was detected also in human fetal lung perfusates. Reverse-phase HPLC analyses of fetal lung extracts suggest that the 126 amino acid ANF prohormone could be one of the ANF forms found in this organ. Although these results need further confirmation by amino acid analysis, they strongly suggest that ANF is synthesized within lung tissues and is not of cardiac origin. This hypothesis is supported by the demonstration of ANF messenger RNA in rat lungs and by our recent report that clearly showed that irANF was secreted by rat lungs and that the 126 amino acid prohormone was present in lung tissues.

The physiological function of lung ANF is still unclear. Like many other hormones and autacoids,
ANF is synthesized by the lungs and could be involved in the modulation of a wide spectrum of activities relevant to the control of blood pressure and water and salt homeostasis.\textsuperscript{11} Sakamoto et al.\textsuperscript{7} showed a correlation between the concentration of lung and plasma irANF and suggested a physiological role for ANF in pulmonary function. Because of the strategic position of the lungs, it is possible that the ANF originating from the right side of the heart may be removed from the circulation. The pulmonary circulation was previously shown to metabolize a number of peptides, including angiotensin I and bradykinin, and other autacoids such as prostaglandins and serotonin (reviewed in Reference 12). We have found that human fetal lung circulation inactivates, to a great extent, bradykinin, angiotensin I, prostaglandin E\textsubscript{2}, acetylcholine, and serotonin.\textsuperscript{13} Recently Turrin and Gillis\textsuperscript{14} reported that 67% of the iodinated ANF-(99-126) injected into the pulmonary circulation of the rabbit was removed in a single pass. These findings suggest a role for the lungs in the control of plasma levels of this factor. Such may not be the case in the fetus, where only 10% of venous return passes through the pulmonary circulation, with the remainder crossing the ductus arteriosus. Although ANF may be an important piece of the fetal hormonal system, it appears that the pulmonary circulation is less involved in controlling its plasma levels.

irANF was also reported in human and rat fetal heart and in umbilical cord plasma.\textsuperscript{15-17} Because of its presence at the embryonic stage, it was suggested that ANF may be a primitive peptide.

In conclusion, our study suggests that ANF is synthesized and released by human fetal lungs. Its role in the physiology and development of the fetus suggests that it contributes to early pulmonary functions; however this remains to be elucidated fully.

Acknowledgments
The authors acknowledge the excellent technical assistance of Bernard Groleau and Micheline Vachon, and the secretarial assistance of Lise Dion.

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Hypertension. 1988;11:I62
doi: 10.1161/01.HYP.11.2_Pt_2.I62

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/11/2_Pt_2/I62

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