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Atrial Natriuretic Polypeptide in the Fetal Rat: Ontogeny and Characterization[1]

LAWRENCE M. DOLAN AND DOUGLAS J. DOBROZSI

Division of Endocrinology, Children's Hospital Medical Center, Cincinnati, Ohio 45229

ABSTRACT. The presence of immunoreactive atrial natriuretic polypeptide (ANP) has been demonstrated in fetal atria early in gestation but further definition of fetal ANP has not been reported. To characterize the principal molecular forms of fetal ANP and to compare fetal ANP to that of the adult of the same species, we extracted the atria of pregnant adult and 20-day fetal rats, the hearts of 14-day fetuses, and intact 12-day fetuses in 1 M acetic acid. Tissue collected from littermates was pooled. We measured ANP by radioimmunoassay before and after gel filtration on Sephadex G-75 in each group. ANP concentrations ($\bar{x}$ ± 1 SD) in ng/mg protein and ng/animal were 1296 ± 505 and 7707 ± 1877 in adult atria (n = 17), 174 ± 44 and 62 ± 13 in 20-day fetal atria (n = 7), and 33 ± 5.3 and 3.7 ± 0.9 in 14-day fetal hearts (n = 6), respectively. Acid extracts from intact 12-day fetuses did not dilute in parallel to the standard curve; therefore, concentrations of ANP for the 12-day fetuses are not reported. ANP concentration rose from the 20-day fetus to the adult ($p < 0.0001$). The major species of ANP eluting from the Sephadex column had an apparent molecular weight of 16 K in all groups. We conclude: 1) ANP is present in the fetus shortly after the completion of organogenesis; 2) 16 K ANP is the principal intracardiac species in the fetus and the adult; and 3) the existence of ANP soon after cardiac development suggests a possible role for ANP in fetal blood pressure and sodium and water homeostasis. (*Pediatr Res* 22: 115–117, 1987)

Abbreviations

ANP, atrial natriuretic polypeptide
RIA, radioimmunoassay
BSA, bovine serum albumin

ANP, a substance synthesized and secreted by mammalian cardiac atria, has potent natriuretic, diuretic, vasodilatory, and antimineralocorticoid properties (1–7). These characteristics suggest that ANP significantly contributes to sodium homeostasis, water balance, and control of systemic blood pressure (1, 8). Three distinct forms of ANP have been documented in adult and immature animals: 1) a 19 K precursor compound; 2) a 13–15 K form, the principal intracardiac species; and 3) a 3 K compound (αANP), the major circulating species of ANP (9–12). The presence of immunoreactive ANP has been demonstrated in fetal atria early in gestation but further definition of fetal ANP has not been reported (13).

To characterize the principal molecular forms of fetal ANP and to compare fetal ANP to the adult of the same species, we extracted ANP from pregnant adult and fetal (20, 14, and 12 day) Sprague Dawley rats (Harlan, Indianapolis, IN) and measured the concentration of ANP by direct radioimmunoassay before and after gel filtration.

MATERIALS AND METHODS

Extraction of ANP from tissue. Timed pregnant (20, 14, and 12 day) Sprague Dawley rats were sacrificed by cervical dislocation or decapitation. The hearts of the adult animals and 20-day fetuses were removed and the atria dissected free. The hearts of 14-day fetuses were harvested and processed intact. The 12-day fetuses were removed from the amniotic cavity and handled without further dissection. Atrial tissue from adult animals was processed individually while the fetal tissue of littermates was pooled. All tissue was snap frozen in liquid nitrogen and stored at −70° C until extraction.

While the tissue was still frozen, 2 ml of hot (100° C) 1.0 M acetic acid was added to each adult or pooled littermate specimen. The samples were then homogenized for 60 s (Tissumizer, Tekmar, Cincinnati, OH), placed in boiling water for 10 min, cooled on ice, and centrifuged at 30,000 × g for 30 min at 4° C. The supernatant was removed, a sample lyophilized for RIA and the remainder stored at −70° C for gel filtration and protein determination. To compare the recovery of immunoreactive ANP among the acid extracts from 14-day fetal hearts and 20-day fetal and adult atria, samples from each tissue were homogenized in 1.0 M acetic acid and divided into two equal volumes. One of the samples was spiked with a known concentration of α-hANP which was approximately three times the amount of ANP present in the tissue sample. Spiked and unspiked samples were then processed as previously described and the amount of ANP documented by RIA. The recovery of α-hANP was calculated by dividing the difference between immunoreactive ANP in the spiked and unspiked samples by the amount of α-hANP added to the spiked sample.

Protein concentration was determined by the method of Lowry *et al.* (14) using a BSA standard (Sigma, St. Louis, MO).

Gel filtration. Acid extracts of adult and 20-day fetal atria, 14-day fetal hearts, and 12-day intact fetuses were chromatographed at 4° C on a 65 × 1.6 cm column of Sephadex G-75, fine grade (Pharmacia, Piscataway, NJ) in 1.0 M acetic acid plus 0.1 mg/ml BSA (Sigma type 7368, St. Louis, MO). One ml of extract was applied and eluted from the column at 4.5 ml/h into 1.5-ml fractions. These samples were lyophilized, reconstituted in RIA buffer, and the concentration of ANP determined by direct RIA. The molecular weight of ANP peaks was determined by comparing their elution positions with those of BSA (66 K), carbonic

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Correspondence and reprints L. M. Dolan, M.D., Division of Endocrinology, Children's Hospital Medical Center, Elland and Bethesda Avenues, Cincinnati, OH 45229.
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anhydrase (29 K), cytochrome C (12.4 K), aprotinin (6.5 K), insulin (6 K), and ANP_{8-33} (2.8 K).

RIA. Lyophilized tissue extracts and G-75 filtration fractions were reconstituted in RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.1% BSA, 0.1% Triton X-100, 0.01% sodium azide, pH 7.4). RIA was performed using the double antibody method described by Tang *et al.* (15). The final dilution of ANP antisera was 1:80,000. Rabbit anti-hANP, ^{125}I-hANP, α-hANP standard, and antirabbit γ-globulin were obtained from Peninsula Laboratories (Belmont, CA). The cross-reactivity of rabbit anti h-ANP is 100% with rat ANP (Ile^{12} α-hANP), 100% with rat atriopeptin III, 5% with rat atriopeptin II, and 0% with rat atriopeptin I. The sensitivity of the assay in our laboratory is 4 pg/tube. At 50% displacement our intraassay coefficient of variation is 4.1% (n = 7) and our interassay coefficient of variation is 10% (n = 6).

Due to the technical difficulty of isolating atria from 14- and 12-day-old fetuses, fetal hearts (14 day) and whole fetuses (12 day) were used for extraction. All samples diluted in parallel to the standard curve of the RIA except for those obtained prior to gel filtration from the 12-day fetuses (Fig. 1). As a result concentrations of ANP in the 12-day fetus are not reported. ANP concentrations are expressed per mg protein and per animal. However, due to a different source of ANP (atrial in adult and 20-day fetus and whole heart in 14-day fetus), statistical analysis was only performed on adult and 20-day specimens. The mean concentration of ANP in the fetal samples was calculated using a value weighted according to litter size. An analysis of variance was employed to compare 20-day fetal and maternal values with fetal samples weighted according to litter size and maternal sample given a weight of one (16).

RESULTS

The recovery of ANP in acid extracts from 14-day fetal hearts and 20-day fetal and adult atria were 98.8 ± 6.0, 83.3 ± 23.2, and 117 ± 10.0% ($\bar{x}$ ± 1 SD, n = 3), respectively. Reported concentrations of immunoreactive ANP are corrected for recovery of α-hANP when the recovery was less than 100%.

The concentrations of immunoreactive ANP ($\bar{x}$ ± 1 SD) are depicted in table 1. The concentration of ANP immunoreactivity increased from the 20-day fetus to the adult whether expressed as ng/mg of protein or ng per animal ($p < 0.0001$).

The gel filtration experiments documented that the molecular weight species of immunoreactive ANP in the extracts of adult and 20- and 14-day fetuses were identical (Fig. 2). Furthermore,

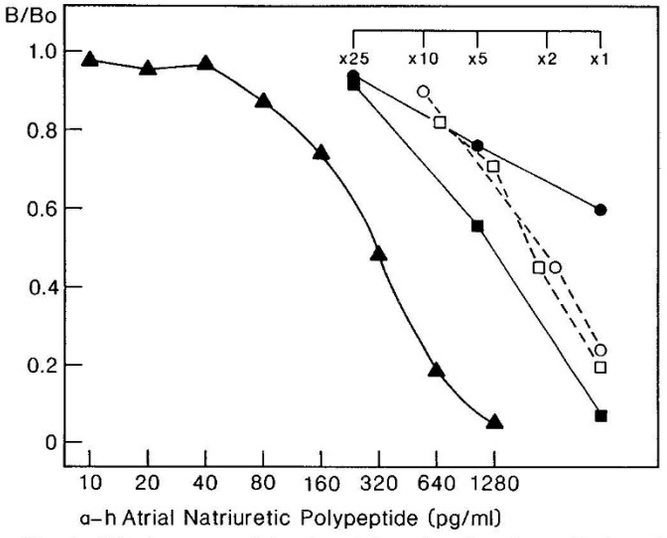

Fig. 1. Dilution curve of ▲, α-h atrial natriuretic polypeptide (standard curve); □, adult atrial extract; ■, 20-day fetal atrial extract; ○, 14-day fetal heart extract; and ●, 12-day whole fetal extract.

Table 1. *Concentration of immunoreactive ANP**

Age (n)	ng/mg protein	ng/animal
Adult (17)	1296 ± 505†	7707 ± 1877†
20-day fetal‡ (7)	174 ± 44	62 ± 13
14-day fetal‡ (6)	33 ± 5.3	3.7 ± 0.9

* ANP immunoreactivity ($\bar{x}$ ± 1 SD) in adult and 20-day fetal atria and 14-day fetal hearts.

† Different than 20-day fetus ($p < 0.0001$).

‡ Pooled littermates.

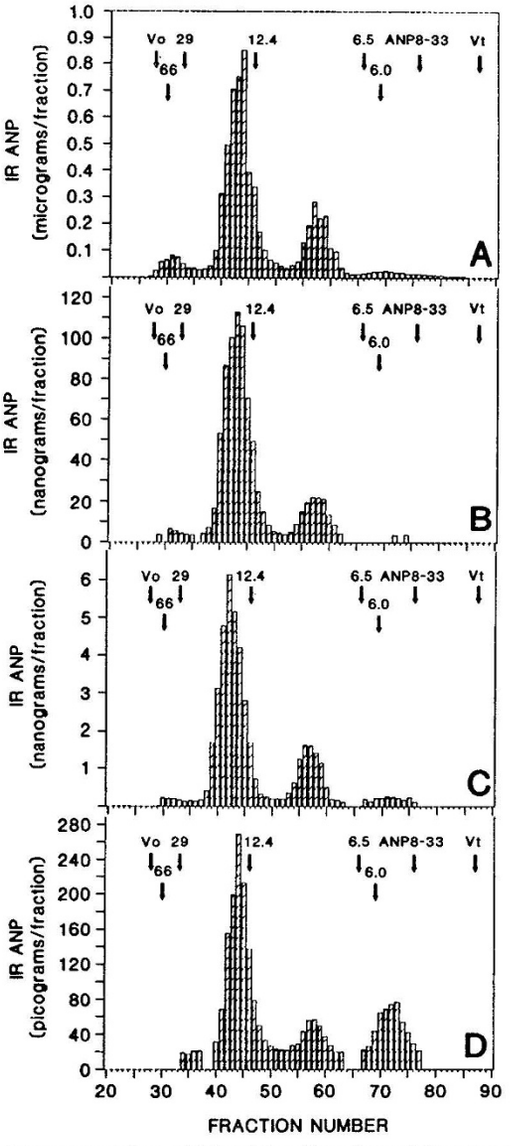

Fig. 2. Immunoreactive atrial natriuretic polypeptide concentration in gel filtration fractions from maternal atria (*A*), 20-day fetal atria (*B*), 14-day fetal hearts (*C*), and 12-day whole fetuses (*D*).

the principal species of ANP in all age groups has an approximate molecular weight of 16 K. A second peak of immunoreactivity was identified in all age groups with an approximate molecular weight of 9 K. Finally, a peak of immunoreactive ANP corresponding with 3–6 K ANP was identified in the 12-day-old fetuses.

DISCUSSION

These data document the presence of immunoreactive ANP in the fetus shortly after the completion of organogenesis. In

addition, the principal intracardiac species of ANP in the adult and 20- and 14-day-old fetus has an apparent molecular weight of 16 K. These results are in agreement with previous reports (9–11) using adult rat and human atria which suggested that the principal intraatrial species has a molecular weight of 13–15 K. The chromatogram from the 12-day fetus also documents a predominance of 16 K ANP. The use of intact fetuses prevents localization of the source of 16 K ANP. However, no study has documented an extraatrial orgin for high molecular forms of ANP suggesting that the source of 16 K ANP in the 12-day fetus is also the atrium.

A second peak of immunoreactive ANP was identified in all ages with an apparent molecular weight of 9 K. These data are in agreement with other studies (17, 18) which also identified 9 K immunoreactive ANP in atrial extract. While the significance of this form of ANP remains to be determined, potential explanations of this finding include the possibility that 9 K ANP is a degradation product of precursor ANP induced by our experimental design and has no physiological significance; that 9 K ANP is a trimer of αANP; and that 9 K ANP is an intermediate form of ANP in the processing of the precursor compound to the circulating form of ANP.

A third immunoreactive peak of ANP with an apparent molecular weight of 3–6 K was identified only in the extract of 12-day, intact fetuses. The significance of this peak is unclear. It may be the result of proteolytic digestion of 16 K ANP by proteases in the fetal homogenate. However, the reports of a 5 K antiparallel dimer of αANP (19) and the presence of 3 K ANP in both fetal hematopoetic tissue of the liver (20) and fetal hypothalamic cells (21) suggest that this peak may also represent either 5 K ANP or αANP extracted from the blood or target organs in the fetus.

In summary the data presented document the presence of immunoreactive ANP in the fetus shortly after organogenesis. The concentration of intraatrial ANP increased from the 20-day fetus to the adult. The principal ANP species in all ages studied has a molecular weight of 16 K. The existence of ANP soon after cardiac development suggests a possible role for ANP in fetal blood pressure and sodium and water homeostasis.

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REFERENCES

1. Ballerman BJ, Brenner BM 1985 Biologically active atrial peptides. J Clin Invest 76:2041–2048
2. Chapeau C, Gutkowska J, Schiller PW, Milne RW, Thibault G, Garcia R, Genest J, Cantin M 1985 Localization of immunoreactive synthetic atrial natriuretic factor (ANF) in the heart of various animal species. J Histochem Cytochem 33:541–550
3. Weidman P, Hasler L, Gnadinger MP, Lang RE, Uehlinger DE, Shaw S, Rascher W, Reubi F 1986 Blood levels and renal effects of atrial natriuretic peptide in normal man. J Clin Invest 77:734–742
4. Kihara M, Nahayama K, Nakao K, Sugawara A, Morii N, Sakamoto M, Suda M, Shinokura M, Kiso Y, Imura H, Yamori Y 1985 Accelerated natriuresis induced by synthetic atrial natriuretic polypeptide in spontaneous hypertensive rats. Clin Exp Hypertens [A]7:539–551
5. Kleinert HD 1984 Atrial natriuretic factor inhibits angiotensin, norepinephrine and potassium-induced vascular contractibility. Hypertension 6:1143–147
6. Yasugima M, Abe K, Kohzuki M, Manno M, Kasai Y, Sato M, Omata K, Kudo K, Tsunoda K, Takeuchi K, Yoshinaga K, Inagama T 1985 Atrial natriuretic factor inhibits hypertension induced by chronic infusion of norepinephrine in conscious rats. Circ Res 57:470–474
7. Kudo T, Baird A 1984 Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor. Nature 312:756–757
8. Dillingham MA, Anderson RJ 1986 Inhibition of vasopressin action by atrial natriuretic factor. Science 231:1572–1573
9. Bloch KD, Scott JA, Zisfein JB, Fallon JT, Margolies MN, Seidman CE, Matsueda GR, Homcy CJ, Grahman RM, Seidman JG 1985 Biosynthesis and secretion of proatrial natriuretic factor by cultured rat cardiocytes. Science 230:1168–1171
10. Miyata A, Kangawa K, Toshimori T, Hatch T, Matsuo H 1985 Molecular forms of atrial natriuretic polypeptides in mammalian tissues and plasma. Biochem Biophys Res Commun 129:248–255
11. Vuolteenaho O, Arjamaa O, Ling N 1985 Atrial natriuretic polypeptides (ANP): rat atria store high molecular weight precursor but secrete processed peptides of 25–35 amino acids. Biochem Biophys Res Commun 129:82–88
12. Thibault G, Lazure C, Schiffrin EL, Gutkowska J, Chartier L, Garcia R, Seidah NG, Chretien M, Genest J, Cantin M 1985 Identification of a biologically active circulating form of rat atrial natriuretic factor. Biochem Biophys Res Commun 130:981–986
13. Morinaga S, Taumuraya M, Nakajima T, Shimosato Y, Yoshinaga K, Yamaguchi K, Abe K, Ohno H, Kanai A 1985 Immunohistochemical and immunocytochemical location of atrial natriuretic polypeptide in human adult and fetal hearts. Acta Histochem Cytochem 18:605–613
14. Lowry OH, Rosebrough NJ, Fan AL, Randall RJ 1951 Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
15. Tang J, Fei H, Xie CW, Suen MZ, Han JS 1984 Characterization and localization of atriopeptin in rat atrium. Peptides 5:1173–1177
16. Winer BJ 1971 Statistical Principles in Experimental Design. McGraw-Hill, New York, pp 417–419
17. Cantin M, Genest J 1985 The heart and the atrial natriuretic factor. Endocr Rev 6:107–127
18. Davies PL, de Bold AJ, deBold ML, Flynn TG, Kennedy B 1985 Main forms of immunoreactive natriuretic factor in atrial tissue and in isolated special atrial granules. Proc Can Fed Biol Soc 28:164(abstr)
19. Kangawa K, Fukuda A, Minamino N, Matsuo H 1984 Purification and complete amino acid sequence of beta-rat atrial natriuretic polypeptide (β-rANP) of 5000 daltons. Biochem Biophys Res Comm 119:933–940
20. Currie MG, Simson JAV, Thompson RP 1986 Evidence for atrial peptide localization in hematopoietic cells. J Cardiovasc Pharmacol 8:1329
21. Morgan K, Lewis M, Scanlon MF, Mir MA 1986 Fetal rat hypothalamic cells release a sodium transport inhibitor, a pro-inhibitor and atrial natriuretic peptide. J Cardiovasc Pharmacol 8:1331