

Detection of atrial natriuretic peptide and its receptor in marginal cells and cochlea tissues from the developing rats

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Abstract

OBJECTIVE: Atrial natriuretic peptide (ANP) regulates the homeostasis of body fluid and blood pressure as a neuropeptide in the central nervous system. To assess the possible physiological role in the inner ear, we investigated the expression of ANP in primary culture of marginal cells, and then we detected the expression of ANP and its receptor (NPR-A) in cochlear tissues derived from neonatal and adult rats of various ages.

METHODS: Marginal cells were isolated from cochlear stria vascularis of the neonatal rats. The cultured cells were subsequently characterized by morphology, immunocytochemistry, and scanning electron microscope (SEM) analysis. In order to examine the expression of ANP in marginal cells as well as in the developing rats' cochleae, immunocytochemistry and reverse-transcription polymerase chain reaction (RT-PCR) were applied respectively.

RESULTS: The present study demonstrated typical characteristics of marginal cells, including cobblestone, polygonal monolayer, pleomorphic growth pattern and the expression of cell type-specific marker, and SEM analysis. Subsequently, immunoreactive product of ANP were found in cultured marginal cells. The mRNAs encoding ANP and NPR-A receptor was expressed in rats' cochleae from postembryonic stage to early postnatal period. During the maturation stage, ANP expression was gradually down-regulated, while the expression of NPR-A receptor was relatively stable.

CONCLUSION: ANP might be synthesized and secreted by marginal cells of stria vascularis, and could play an important role in modulating the microenvironment of the inner ear. In addition, ANP might contribute to development and growth process of cochlea.

INTRODUCTION

Hearing impairment is the most prevalent form of sensory deficit, which has a significant impact on the quality of human's life. It is well known that the maintenance of the homeostasis of the inner ear plays an important role in normal hearing (Zhao *et al.* 2006), which requires the regulation of the inner ear fluid systems, i.e., perilymph and endolymph. Many structures in the cochlea are involved in the regulation of inner-ear homeostasis and one of the most important is the stria vascularis, which is responsible for endolymph secretion, while its impaired function is related to various inner-ear diseases (Ciuman 2009). The stria vascularis is a multilaminar epithelium containing at least three cell types, including marginal cells, basal cells and intermediate cell (Slepecky 1996). Although they all provide contribution to the regulation of inner ear's function, there is strong evidence that marginal cells play a vital role in inner ear physiology, because marginal cells may secrete K^+ into the endolymph, which play a key role in keeping stabilization of inside environment of the inner ear (Quraishi & Raphael 2007). Therefore, it is important for an in-depth investigation of the function of the marginal cells in the cochlea. Until now, there was no direct evidence to prove whether the marginal cells had a paracrine or autocrine function for the regulation of labyrinthine fluid.

ANP is a potent endogenous diuretic and natriuretic substance and is synthesized and secreted by the cardiac atria, which has been shown to improve renal and cardiac function (Goetz 1988; Nakamoto *et al.* 1987). This hormone mediates natriuresis and vasodilatation through its specific receptors, which are known to exist in many organs except mainly in kidney (Brenner *et al.* 1990). In addition, it was reported that ANP could play a role in maintenance of homeostasis in the inner ear (Genest & Cantin 1988). Though not being in the same functional system, many evidences suggest a strong similarity between the inner ear and kidney, including anatomical configuration, morphological similarities, pharmacological responses to certain drugs, and even some pathological states (Arnold 1976; Tago & Yanagita 1992; Holzmüller 2000). All these supported a viewpoint that there existed close relationship between the kidney and inner ear. Recently, it had been reported that ANP may be associated with improved outcomes when used in low doses for preventing acute kidney injury (AKI) and may reduce long-term mortality as well as the initiation of dialysis after coronary angiography and intervention (Nigwekar *et al.* 2009a; Nigwekar *et al.* 2009b). Therefore, we lead to the hypothesis that ANP might be participate in protection from a variety of stresses by the regulation of homeostasis in the inner ear.

Although Meyer zum Gottesberge & Lamprecht (1989) first reported that ANP bound to the lateral wall of the cochlea, direct evidence for the existence of ANP

in the stria marginal cells is still lacking. Additionally, it had been proved that ANP was an important tool for molecular and developmental biologists. Expression of ANP was an early and specific marker for the differentiating working myocardium of the developing heart (Houweling *et al.* 2005) But their roles in the developing inner ear also remain to be determined.

In the present study, marginal cells were isolated from cochlear stria vascularis of the neonatal rats and were subsequently characterized by morphology, immunocytochemistry, and SEM analysis. Furthermore, we firstly demonstrated the expression of ANP in marginal cells by immunocytochemistry. Then, the mRNA encoding ANP and NPR-A receptor was determined in the developing rats' cochleae by RT-PCR.

MATERIALS AND METHODS

The procedures concerning animals reported in this study were approved by the Animal Care and Use Committee at the Fourth Military Medical University. The Sprague-Dawley (SD) rats used in the experiments were provided by the animal centre of the Fourth Military Medical University.

Isolation of cochlea-derived marginal cells

SD rats (postnatal day 1–3) were anesthetized with 2% sodium pentobarbital (2 ml/kg; Sigma, USA) and sacrificed to remove the temporal bones and the bony capsule. Briefly, the lateral wall of the cochlea was exposed, and the stria vascularis was separated from the spiral ligament. Then, the stria vascularis of each cochlear turn was transferred to a cell-tak (BD Biosciences, Bedford, USA) coated coverslip in a culture dish (35 mm diameter; Sigma). These tissues were cultivated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere and observed every day under an inverted light microscope.

Immunocytochemistry

For immunohistochemistry (Han *et al.* 2009), the isolated cells were fixed in 4% paraformaldehyde (pH 7.4) and rinsed three times with PBS for 10 min. After rinsing, the cells were permeabilized by a 5-min exposure to 0.3% Triton X-100 at room temperature. The cells were then washed with PBS three times for 10 min and incubated with blocking solution (5% normal horse serum) for 30 min at room temperature. The cultured cells were then incubated overnight at 4°C with monoclonal antibodies specific to CK18 (1:100; Chemicon) or monoclonal mouse anti-ANP (1:150; Chemicon) Cells were washed three times for 5 min each with PBS, and then incubated with horseradish peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin antibodies (Envision + detection system, K4007, DAKO). The CK18 and ANP localization was revealed by using a peroxidase reaction with DAB + (3,3-diaminobenzidine

tetrahydrochloride). After completion of the immunostaining cycle, slides were dehydrated with sequential ethanol washes, and coverslipped for microscopic evaluation under the Olympus Microscope (Olympus Co. Ltd., Tokyo, Japan). Negative controls were performed in parallel, in which the cells were treated with a solution of 1% BSA (in PBS) without a primary antibody. The protocols were repeated using monolayers from several different generations to assess reproducibility of the immunoreactivity for each antiserum.

Scanning electron microscope

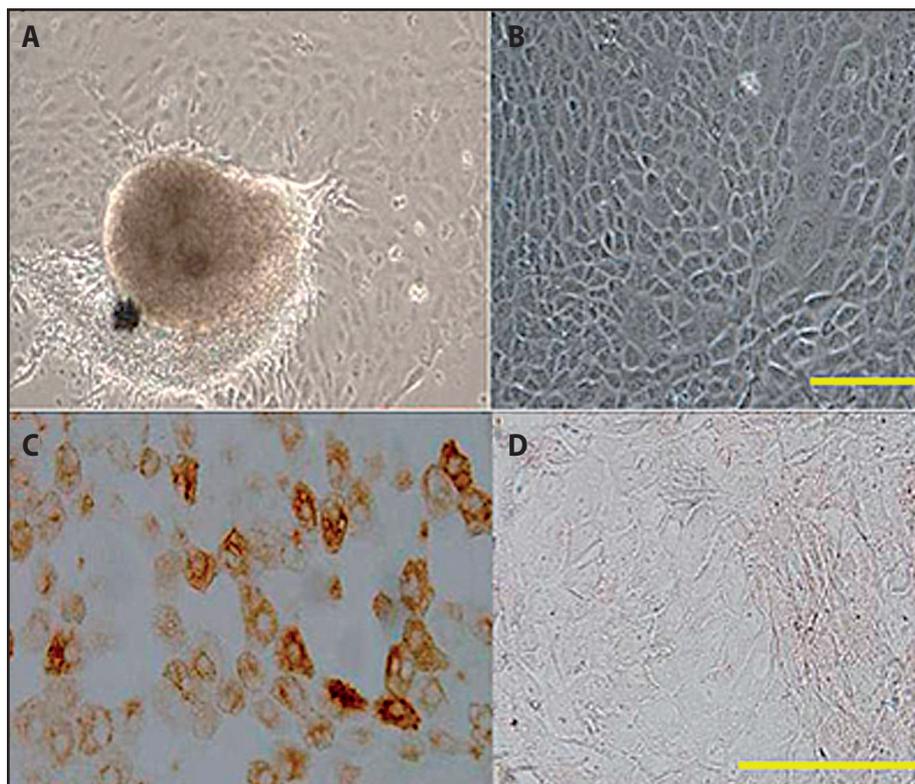
The cells derived from the third passage were fixed on plastic culture dishes with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4) at 4°C for 2 h. The plates were rinsed with 0.1 M PBS and were post-fixed in 1% osmium tetroxide for 2 h, and then were put in 2% tannic acid two times, each for 30 min. Then the dishes were dehydrated through a graded ethanol series. After absolute ethanol, they were dried in a critical point drier (HCP-2, Hitachi). Specimens were fixed on a metal stage, gold-coated in a sputter coater (E102 Ion Sputter, Hitachi) and observed under a SEM (Hitachi S-800).

RT-PCR

Embryonic day 14 (E14), postnatal day 1, 3, 6, 8 (P1, 3, 6, 8), and adults SD rats were deeply anesthetized with 2% sodium pentobarbital (2 ml/kg; Sigma, USA). Whole cochleae without vestibular organs were collected. Total RNA was extracted using the Total RNA

Isolation System (Promega). cDNA synthesized with RevertAid™ First Strand cDNA Synthesis Kits (Fermentas) was amplified by PCR. PCR amplification for ANP comprised of (1) 94°C for 5 min, (2) 60°C for 1 min, and (3) 72°C for 7 min, was conducted for 30 cycles using 1 µl of cDNA templates. The conditions for the NPR-A were as follows: 5 min hot start at 94°C, followed by 30 cycles of 1 min at 55°C, 30s at 72°C, and 7 min at 72°C. We designed primers of 790 base pairs (bp) specific for ANP: sense primer, 5'-GCCGGTAGAAGATGAGGTCA-3'; antisense primer, 5'-GGGCTCCAATCCTGTCAATC-3'. These primers were expected to amplify a cDNA fragment of 269bp. Another primer of 4068 bp specific for NPR-A receptor: sense primer, 5'-GAGA-ACAGCAGCAACATCCT-3'; antisense primer, 5'-AGCGCACAGCATCCAGTAGT-3'. These primers were expected to amplify a cDNA fragment of 454bp. GAPDH, forward: 5'-GTATGACTCTACCCACGGCAAGTTC-3', reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. We compared the relative quantification of the target ANP and NPR-A mRNA transcript to a reference GAPDH mRNA transcript. The expression level of ANP and NPR-A mRNA was equivalent to the ratio of band intensity of ANP or NPR-A and GAPDH mRNA. Statistical analysis was performed with SPSS for Windows Version 12.0.1 (SPSS Inc.) using One-way ANOVA. The ANP and NPR-A PCR product was sequenced by 3730xl DNA Analyzer (Applied Biosystems).

Fig. 1. Characterisation of marginal cells of stria vascularis by immunohistochemical staining for CK18. A. Isolated marginal cells from the explants of stria vascularis after two days of culture. B. Marginal cells showed a "cobblestone-like" appearance. C. The expression of CK18 staining in the marginal cells. The positive reaction was prominent along the cell plasma lemma or regions of cell-cell contact. D. As for the negative control, no immunostaining signal was detected in marginal cells of stria vascularis. Scale bar=100 µm.



RESULTS

Isolation and characterisation of marginal cells

After cultured for three days, the morphology of marginal cells was evident with characteristic of pleomorphic though the majority was small and spindle-shaped. Upon confluence, a cobblestone, polygonal monolayer, characteristic of cultured epithelial cells was evident (Figures 1A, B). After cultured for three weeks, the cultured marginal cells showed uniform immunostaining for CK18, and the staining was prominent along the cell plasma lemma or regions of cell-cell contact. The negative control showed negative immunostaining (Figures 1C, D).

Ultrastructural structure of marginal cells

As shown by SEM, the cells grew in a spread monolayer way, with tight cell-cell contact (Figures 2A, B). Abundant microvilli were on the surface of the cultured cells (Figure 2C), which was consistent with the marginal cells of human's cochlea. Additionally, there were large quantities of vesicle-like secretion substance (Figure 2D).

Immunocytochemistry

The ANP expression was detected in the marginal cells of stria vascularis by immunohistochemical analysis. The results showed that ANP was expressed mainly in cytoplasm, especially around the nucleus (Figure 3).

The negative control showed negative immunostaining (data not shown).

RT-PCR in the marginal cells and cochlea tissues

After PCR amplification of cDNAs using ANP and NPR-A specific primers in the cochlea tissues of different ages, sharp bands of the expected size were observed on the gel (Figure 4A). The results showed that a band of the same size of ANP were amplification in the cochlea tissues from E14, P1, P3 rats, and the expression levels were no statistic significance ($p>0.05$). However, the faint band of ANP was observed in the P6 rats, and there was a complete absence after P8 ($p<0.05$). In addition, the strong bands of NPR-A were observed in the rats' cochlea from postembryonic stage to early postnatal and adult period, and there were no statistic significance among them ($p>0.05$). The sequence of PCR product was determined to be identical to the originally published sequence of ANP (GenBank accession no. NM_012612) and NPR-A (GenBank accession no. NM_012613). To assess the expression level of ANP and NPR-A mRNA among the rats' cochlea of different ages, a comparison of the ratio of band intensity of ANP or NPR-A mRNA and GAPDH mRNA was made in Fig. 4B. The ratio of ANP mRNA and GAPDH mRNA was markedly decreased, and the difference was significant ($p<0.05$). The ratio of NPR-A mRNA and GAPDH mRNA was relatively stable.

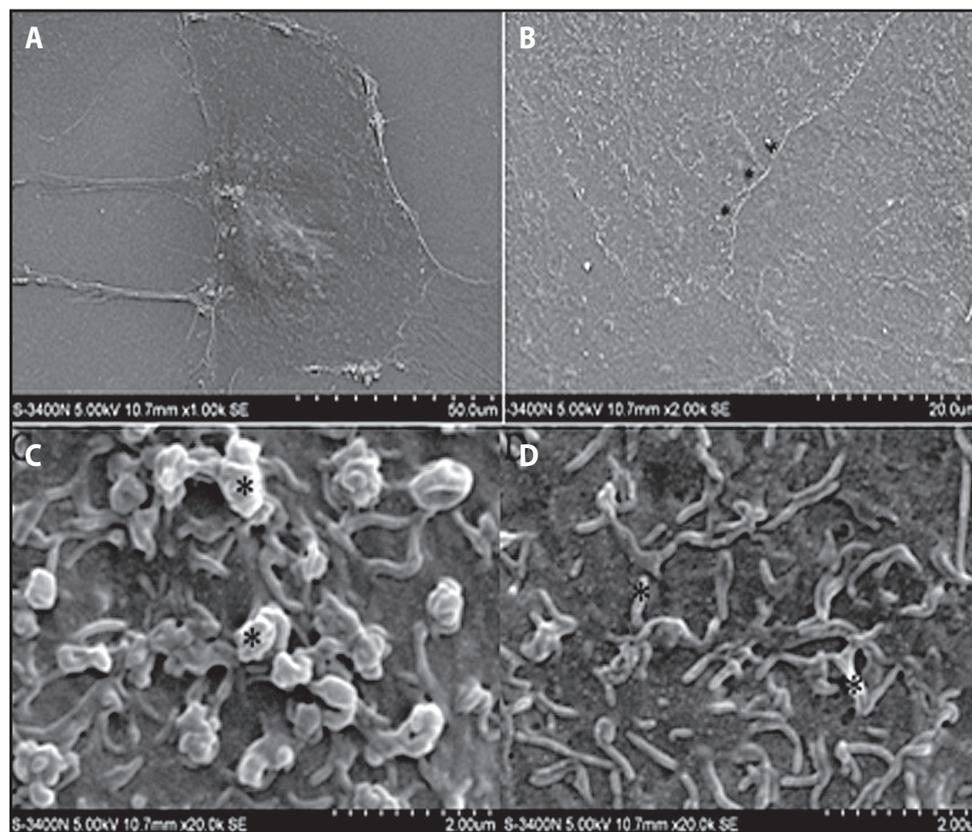


Fig. 2. The ultrastructural structure of the cultured marginal cells. **A.** A marginal cell grew in a spread monolayer way. **B.** Tight cell-cell contact (asterisk). **C.** Abundant microvilli on the surface (asterisk). **D.** Large quantities of vesicle-like secretion substance (asterisk).

DISCUSSION

It is necessary for normal auditory function that maintenance the homeostasis of the inner ear. A variety of regulatory mechanisms, such as a blood-labyrinth barrier, might participate in this process. The blood-labyrinth barrier was assumed to be very important for physiological and pathophysiological process in the cochlea (Ermisch 1992; Suzuki *et al.* 2000). Any disturbance in this barrier can serve as a triggering mechanism for abrupt functional disturbances of inner ear fluid homeostasis and might lead to sensorineural hearing loss. The stria vascularis, which has a role in separating the endolymph from perilymph, contains three special cell types and two epithelial barriers (Wangemann 2002). Facing the endolymph, the strial marginal cells form the integral tight junction, and the impairment of the tight junction may lead to different sensorineural deafness (Riazuddin *et al.* 2006). Additionally, marginal cells are directly responsible for the high endolymphatic K^+ state. Therefore, it is important for an in-depth investigation of the function of the marginal cells. In the present study, we had successfully established a primary culture of marginal cells from rats *in vitro*. Moreover, the characteristics of these cultured cells were typical, including cobblestone, polygonal monolayer, pleomorphic growth pattern and the expression of cell type-specific marker.

Natriuretic peptides (NPs) are cardiovascular cyclic peptide hormones with diuretic, natriuretic and vasodilatory properties (Levin *et al.* 1998). There are three subtypes of natriuretic peptide: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Imura *et al.* 1992). ANP is synthesized and secreted from the cardiac atria, and is used in the treatment of various disorders, including hypertension, renal insufficiency, and also play a role in the regulation of inner ear fluid (Chen & Burnett 2006; Suga *et al.* 1992). Both ANP and BNP can inhibit renin and aldosterone secretion by binding NPR-A, which is important for sustaining ion homeostasis (Suga *et al.* 1992). However, the expression of the endogenous ANP/NPR-A system in marginal cells of stria vascularis and in the process of inner ear development is not fully understood. Our results indicated that immunoreactive product of ANP was identified in the cytoplasm of the cultured marginal cells of stria vascularis. Cell culture techniques have advantages of elucidating cell function, but their limitations, such as lack of endocrine and paracrine pattern *in vivo* system, further studies are needed to clarify the functions of ANP/NPR-A system in the inner ear *in vivo*.

It was reported that the onset of hearing occurred around 9–12 days after birth in the rat (Rube 1978). The present study showed both mRNAs encoding ANP and NPR-A receptor were expressed in rats' cochlea from postembryonic stage to early postnatal period. Therefore, we considered that ANP and NPR-A recep-



Fig. 3. Expression of ANP in marginal cells of stria vascularis. The positive reaction was expressed mainly in cytoplasm and around the nucleus. Scale bar=100 μ m.

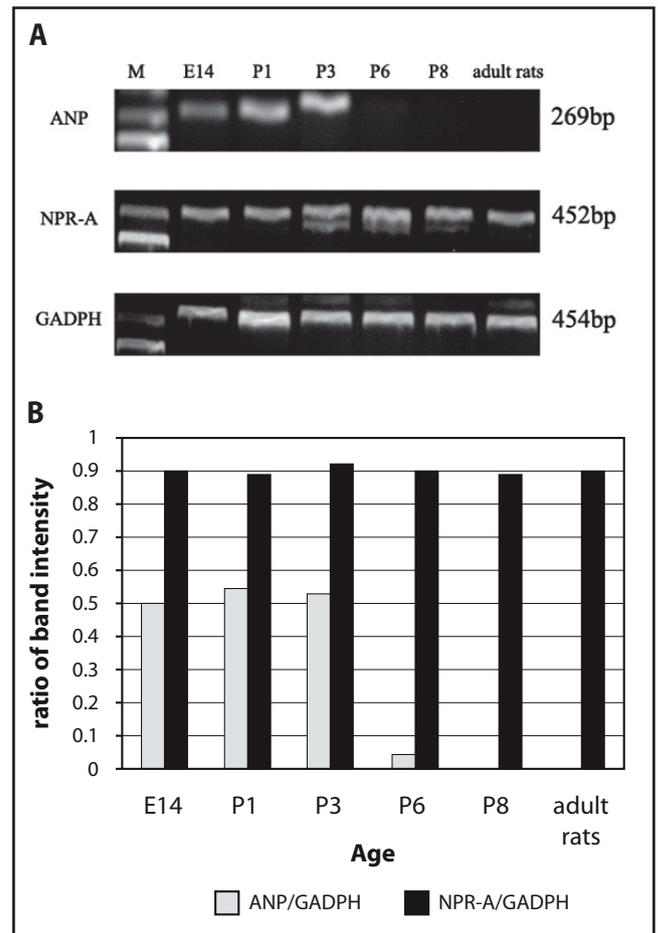


Fig. 4. RT-PCR. **A.** ANP and NPR-A mRNA levels in cochlea tissues of embryonic day 14 (E14), P1, P3, P6, P8 and adult rats. M: DNA marker DL2000; **B.** A comparison of the ratio of band intensity of ANP or NPR-A mRNA and GAPDH mRNA among the rats' cochlea of different ages. The ratio of ANP mRNA and GAPDH mRNA was markedly decreased, and the difference was significant ($p < 0.05$). The ratio of NPR-A mRNA and GAPDH mRNA was relatively stable.

tor expressed immediately before the onset of hearing development may be critical for normal inner ear developing. However, during the maturation, ANP expression was down regulated while the expression of NPR-A receptor was relatively stable. The pattern of their expression strongly suggests that ANP and NPR-A receptor may play a role in maintaining the inner ear fluid system, acting in an autocrine and/or a paracrine fashion. However, the mechanisms of this phenomenon need further research. Additionally, it remains to be elucidated whether ANP participates in protection from a variety of stresses in the cochlea.

In conclusion, studies had demonstrated that the expression of ANP/NPR-A system in marginal cells and in the process of cochlear development. We concluded that ANP might play an important role in modulating the microenvironment of the inner ear and contribute to the development process of cochlea. Furthermore, ANP might be considered as a candidate gene for reversing hearing impairment in the future.

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