Detection of atrial natriuretic peptide receptor in the labyrinth of the mouse inner ear

Lili Long[1], Yuedi Tang[1]*, Qingjie Xia[2], Zengliang Xia[2], Jin Liu[3]

1. Department of Otorhinolaryngology; West China Hospital of Sichuan University
2. Department of Ophthalmology; West China Hospital of Sichuan University
3. Department of Anesthesiology and Critical Care Medicine; West China Hospital of Sichuan University, P. R. China

**Correspondence to:* Dr. Yuedi Tang, Department of Otorhinolaryngology, West China Hospital of Sichuan University, No.37 Guo-xue-Xiang, Chengdu, Sichuan 610041, P. R. China.
e-mail: tangyd@hotmail.com

Submitted: 2008-02-12 Accepted: 2008-06-21 Published online: 2008-08-30

Key words: **atrial natriuretic peptide receptor; inner ear; stria vascularis; vestibular organ; reverse transcription-polymerase chain reaction**

Neuroendocrinol Lett 2008; **29**(4):577–580 **PMID:** 18766159 NEL290408A26

Abstract

OBJECTIVES: To examine whether the atrial natriuretic peptide receptor (NPR-A) is present in the secretory regions of the membrane labyrinth of the adult mouse inner ear. **SETTING:** Recent studies have implied that the homeostasis of endolymph fluid in the inner ear may be regulated by receptor-mediated mechanisms. Several studies have identified the presence of atrial natriuretic peptide receptors in the inner ear of guinea pig and rat. As a member of the natriuretic peptide receptor family, which also includes B-type natriuretic peptide receptor (NPR-B) and C-type natriuretic peptide receptor (NPR-C), NPR-A may be involved in the regulation of fluid homeostasis in the inner ear. **METHODS:** In this study, samples of stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue from the ears of 6 adult mice were obtained by immediate excision of bony labyrinth under operating microscope after decapitation. Total RNA was isolated and mRNA was amplified by the reverse transcription-polymerase chain reaction (RT-PCR) using consensus primers flanking a region of 127 bp at the target sequence. Mouse renal cortex known to contain NPR-A was used as a positive control. **RESULTS:** We demonstrated that NPR-A was expressed in the mouse stria vascularis as well as in the nonstrial tissue of the cochlear lateral wall and vestibular organ. **CONCLUSION:** These results suggest that natriuretic peptides may play an important role in maintaining the fluid homeostasis of inner ear endolymph via interaction with NPR-A.

1. INTRODUCTION

The various compartments of the inner ear endolymphatic space are filled with fluids composed of different electrolyte components. Fluid homeostasis in the endolymphatic system is essential for the maintenance of normal function of the inner ear. Once the balance of such homeostasis is disrupted, dysfunction of the inner ear, such as Meniere's disease, more specifically, endolymphatic hydrops, may occur (Sterkers *et al.* 1984). Endolymphatic hydrops is a condition in which the endolymphatic space of the inner ear is distended secondary to a presumed excess of fluid volume or pressure. It is associated with symptoms, such as aural fullness, tinnitus, episodic vertigo, and a fluctuating progressive hearing loss initially in the low tones. Etiologic and therapeutic attempts to unravel the mystery of Meniere's disease have been directed at the volume mismatch of the inner ear. However, the underlying mechanisms of endolymph homeo-

ORIGINAL ARTICLE

Abbreviations
ANP – atrial natriuretic peptide,
NPR-A – atrial natriuretic peptide receptor,
RT-PCR – reverse transcription-polymerase chain reaction,
GAPDH – glyceraldehyde-3-phosphate dehydrogenase

stasis are still largely unknown, particularly the hormonal regulation.

Function of the inner ear is tightly regulated by hormones. The presence of aldosterone receptor, atrial natriuretic peptide (ANP) receptors, vasopressin receptors and endothelin receptors in the inner ear has been reported, suggesting a critical role in hormonal regulation (Furuta *et al.* 1994; Furuta *et al.* 1995; Furuta *et al.* 1998; Yan *et al.* 2007). One of the important hormone candidates involved in the regulation of inner ear fluid homeostasis is ANP, which was first extracted from rat atrial tissue by DeBold AJ (Baines *et al.* 1983). ANP can exert a smooth muscle effect, with vasodilation, diuretic, and natriuretic activities in the kidney. It is a member of the natriuretic peptide family. The natriuretic peptide family consists of five members: ANP, B-type natriuritic peptide, C-type natriuretic peptide, urodilatin and dendroaspis natriuretic peptide. Each of them has its specific biological functions (Cea, 2005). There are three natriuretic peptide receptors, atrial natriuretic peptide receptor (NPR-A), B-type natriuretic peptide receptor (NPR-B) and C-type natriuretic peptide receptor (NPR-C), which can be activated by different natriuretic peptides. ANP exhibits its biological activity through binding NPR-A to increase guanylyl cyclase activity (Potter *et al.* 2006).

NPR-A consists of an extracellular ligand-binding domain for natriuretic peptide binding, a single hydrophobic membrane-spanning region, and an intracellular domain which is made of a kinase homology domain, a coiled-coil dimerization domain, and a guanylyl cyclase catalytic domain. By activating guanylate cyclase, ANP engagement with its receptor increases the concentrations of intracellular cyclic guanosine monophosphate which serves as a second messenger to initiate the cascade reactions needed in biological functions of the cells (Potter *et al.* 2006). The effects of stimulating NPR-A may be different according to the activation of specific signal pathways in different tissues. It can modulate sodium channels and transporters, activate protein kinase GI, GII, or stimulate adenosine 3', 5'-cyclic monophosphate-hydrolyzing phosphodiesterase$_2$ to increase capillary permeability, vasodilation, and natriuresis (Potter *et al.* 2006).

NPR-A mRNA is highly expressed in the kidney, adrenal glomerulosa, adrenal medulla, pituitary, cerebellum and endocardial endothelial cells (Potter *et al.* 2006). Lamprecht and Meyer zum Gottesberge (1988) first reported that ANP receptors were distributed in the inner ear of guinea pig, suggesting a possible role of these receptors in the regulation of labyrinthine fluid composition and volume. Despite this report and several others, available information about the expression of ANP receptors in the inner ear is still limited due to the lack of direct ANP receptor illustration (Meyer *et al.* 1995; Lamprecht & Meyer, 1988; Meyer & Lamprecht, 1989). In addition, the results of previous studies were controversial (Meyer *et al.* 1995; Lamprecht & Meyer, 1988; Meyer & Lamprecht, 1989; Seebacher *et al.* 1999; Koch *et al.* 1992; Furuta *et al.* 1995).

In the inner ear, stria vascularis in the cochlea and the dark cell epithelium in the vestibular organs are believed to be the secretory regions of the inner ear to produce endolymphatic fluid (Arenberg *et al.* 1970), as well as spiral ligament in the nonstrial tissue of the cochlear lateral wall (Sterkers *et al.* 1988). Our present study was designed to evaluate whether NPR-A mRNA was expressed in these three regions of mouse inner ear using reverse transcription-polymerase chain reaction (RT-PCR). We demonstrated the expression of the mRNA encoding NPR-A in the tissues of adult mouse inner ear. This study will lead to further understanding of the physiological role of natriuretic peptides in the inner ear.

2. MATERIALS AND METHODS

2.1 Tissue preparations

The Kunming mouse is an outbreed strain of laboratory animal widely utilized in related pharmaceutical and genetic studies in China. Six healthy adult Kunming mice at the age of about 30 days used in this study were provided by the Animal Care and Use Committee at the Huaxi Medical School of Sichuan University. The research reported here was conducted according to the animal care guidelines established by West China Hospital of Sichuan University. After decapitating the mice and removing the temporal bone, the stria vescularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue in the inner ear were dissected at 4°C as described previously (Marcus & Chiba, 1999). The renal cortex of the adult mice was used as positive control.

2.2 Design of primers to identify mouse NPR-A

We designed pairs of primers for PCR amplification according to the NCBI Genebank of mice (Accession number: ENSMUSP00000029540). The primers were synthesized by Shanghai SAGON. The primers for mouse NPR-A were as follows: forward primer 5'-CGACGGGCTCCTGCTCTAT-3' and reverse primer 5'-CAGGTATCCTGTCACACCTTG-3'(127 bp). The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: forward primer 5'-CCTCAAGATTGTCAGCAAT-3' and reverse primer 5'-CCATCCACAGTCTTCTGGGT 3'(141 bp).

2.3 RNA preparation and cDNA synthesis

Transcripts for NPR-A were assayed by RT-PCR. Total RNA was isolated from the stria vascularis, nonstrial tissue of the cochlear lateral wall, vestibular organ tissue and renal cortex of the adult mice using Trizol

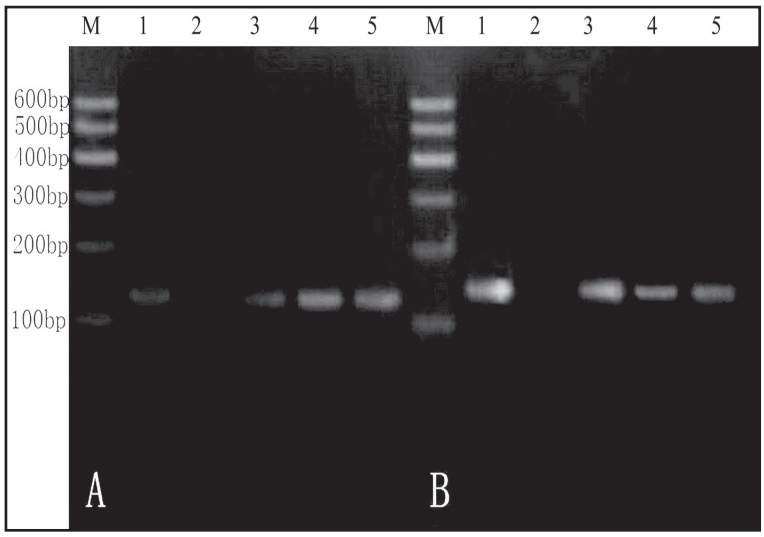

Figure. Ethidium bromide staining of PCR products on a 2% agarose gel from adult mice stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue. NPR-A cDNA of the mouse renal cortex served as a positive control for NPR-A consensus PCR primers (lane 1). The lane 2 in both A (NPR-A) and B (GAPDH) shows negative control without templates. Total RNA preparations from stria vascularis of cochlea (lane 3), nonstrial tissue of the cochlear lateral wall (lane 4) and vestibular organ (lane 5) after reverse transcription were subjected to 45 cycles of PCR. Amplification of NPR-A cDNA is a fragment of 127 bp, and amplification of GAPDH cDNA is a fragment of 141 bp. M indicates the marker.

reagent (MRC USA). RNA was reversely transcribed into cDNA using the Revert Aid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas Inc.) with addition of random hexamer primers. Briefly, 5μl of purified RNA was used as templates for cDNA synthesis in the presence of 1μl M-MLV reverse transcriptase (200U), 1μl of random hexamer primer, 4μl of 5 × reaction buffer, 2μl of hexamer 1 × (Roche), 2μl of dNTP mix (10mM each), and 6μl of RNAse-free water. After incubation for 60 min at 42°C, the reverse transcriptase was inactivated at 70°C for 10 min, and cDNAs were stored at –20°C until further analysis.

2.4 Polymerase chain reaction

The presence of specific message in cDNA derived from total RNA preparations was assayed by amplification using the oligonucleotide primers listed above. NPR-A cDNAs in the renal cortex was used as positive control templates and for adjusting the amplification conditions. PCR without templates was performed as a negative control. PCR for GAPDH was carried out as a control for successful performance of RNA extraction and reverse transcription procedures.

For the primer pairs used in this study, the amplification mixture containing 3μl of 10 × reaction buffer, 3μl of 25mM $MgCl_2$, 0.36μl of 25mM dNTP, 1μl of forward primer, 1μl of reverse primer, 1.5μl of dyes and 1.5U of Taq, and double distilled water was added to get a whole volume of 5μl for each reaction tube.

The PCR was performed as follows: one denaturation cycle for 2 min at 94°C followed by 45 amplification cycles, including denaturation for 20 sec at 94°C, annealing for 20 sec at 58°C, extension for 30 sec at 72°C, and final one extension cycle for 5 min at 72°C. The amplified PCR products were routinely assessed by horizontal electrophoresis in 2% agarose gels containing 1μl/ml of ethidium bromide.

3. RESULTS

The transcripts for NPR-A were detected in the stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue from inner ear membrane labyrinth of adult mouse. Fig. A and Fig. B showed the PCR products amplified by primers for NPR-A and GAPDH, respectively. As shown in Fig. A, the RNAs isolated from the stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue of inner ear gave a single sharp band of 127 bp using the NPR-A-specific primers. Renal cortex sample, which has been previously shown to express NPR-A (Potter *et al.* 2006), was used as a positive control (Fig. A; lane 1). Lane 2 showed the negative control. GAPDH was used as a housekeeping gene. DNA bands at the expected sizes of GAPDH were observed on the agarose gels (Fig. B lanes 1, 3, 4, 5).

4. DISCUSSION

The study has demonstrated the presence of NPR-A transcripts in stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue in the mouse inner ear by a sensitive RT-PCR analysis. The DNA bands on the gel were of expected length suggesting that NPR-A mRNA expression was detected specifically both in the tissue samples of inner ear and that of renal cortex. The PCR products contained no introns, suggesting that there was no genomic DNA contamination, and that the DNA was generated from mRNA transcripts.

NPR-A mRNA was expressed in the stria vascularis. The selective ligand of NPR-A is ANP which plays an important role in maintaining fluid balance. ANP-immunoreactive particles were seen mainly in the cytoplasm of the stria vascularis marginal cells of normal guinea pigs (Chen *et al.* 1994). Studies suggest that ANP may be locally synthesized and activate NPR-A in a paracrine/autocrine manner. The specific signal

transduction following NPR-A activation by ANP in the stria vascularis is largely unknown. Our results suggest that ANP may regulate cochlear endolymph fluid balance via activation of NPR-A.

The secretory structures of the cochlea include stria vascularis and spiral ligament (Tran & Lecain, 2002). In this study, NPR-A mRNA expression was also detected in the nonstrial tissue of the cochlear lateral wall at where spiral ligament is located. The spiral ligament of the cochlear lateral wall exhibited pronounced immunoreactivity to ANP. It was assumed that ANP may participate in the regulation of the water electrolyte balance (Yoon & Hellstrom, 1992). The result of our study supports this assumption.

The dark cells of the vestibular organ are thought to be responsible for the production of the vestibular endolymph because of their morphologic resemblance to the marginal cells of the stria vascularis (Sterkers *et al.* 1988). NPR-A mRNA expression was detected in the vestibular organ tissue, suggesting a similar role as it in the stria vascularis.

ANP is known to interact with vasopressin and mineralocorticoid (Jard, 1983; Brenner *et al.* 1990; Horisberger & Rossier, 1992). Both mineralocorticoid type I receptor and vasopressin receptors were detected in the cochlea (Furuta *et al*. 1994; Furuta *et al*. 1998). It is proposed that ANP may modulate the function of vasopressin and mineralocorticoid in the inner ear to regulate endolymph fluid balance.

The identification of NPR-A in the inner ear is the first step to understand the possible roles of ANP regulation in inner ear fluid homeostasis. Several studies have identified ANP receptors in the inner ear of guinea pig and rat, and have demonstrated their presence in the stria vascularis, spiral ligament in the cochlea and the secretory epithelium of the vestibular organ. However, some of these results are contradictory (Koch *et al.* 1992; Seebacher *et al.* 1999; Meyer *et al.* 1995; Lamprecht & Meyer, 1988; Meyer & Lamprecht, 1989). A few studies detected the existence of ANP receptors or NPR-A in the vestibular organ (Meyer *et al.* 1995; Lamprecht & Meyer, 1988; Meyer & Lamprecht, 1989; Seebacher *et al.* 1999). Conversely, Koch *et al.* (1992) could not detect any ANP receptors in that area. Besides, NPR-A mRNA expression was not observed in the stria vascularis in another study (Furuta *et al.* 1995). In this study, specific bands of NPR-A cDNA product were detected in all the endolymph-producing areas in the inner ear, unlike some previous reports. This may be due to the biological difference between species or different PCR amplification cycles we chose.

In conclusion, mRNA expression of NPR-A is confirmed in the adult mouse stria vascularis as well as in the nonstrial tissue of the cochlear lateral wall and vestibular organ. NPR-A may be involved in fluid homeostasis in the mouse endolymphatic system. These studies provide the evidence to further investigate the mechanism of NPR-A action.

Acknowledgements: The authors thank Mr. Faqiang Zhang and Ms. Yanfang Chen for their technical assistance. This project was supported by National Natural Science Foundation of China (NSFC 30271408 and 30471880 to YD Tang).

REFERENCES

1 Arenberg IK, Marovitz W and Shambaugh GE (1970). The role of the endolymphatic sac in the pathogenesis of endolymphatic hydrops in man. *Acta Otolaryngol Suppl.* **275**: 1–49.
2 Baines AD, DeBold AJ and Sonnenberg H (1983).Natriuretic effect of atrial extract on isolated perfused rat kidney. *Can J of Physiol and Pharmacol.* **61** (12): 1462–1466.
3 Brenner BM, Ballermann BJ, Gunning ME, Zeidel ML (1990). Diverse biological actions of atrial natriuretic peptide. *Physio. Rev.* **70** (3): 665–699.
4 Cea LB (2005). Natriuretic peptide family: new aspects. *Curr Med Chem.* **3** (2): 87–98.
5 Chen HX, Wang JL, Liu QC, Qiu JU (1994). Distribution and location of immunoreactive atrialnatriuretic peptides in cochlear stria vascularis of guinea pig. *Chin Med J.* **107** (1): 53–56.
6 Furuta H, Luo L, Ryan AF, Mori N (1998). Expression of mRNA encoding vasopressin V1a, vasopressin V2, and ANP-B receptors in the rat cochlea. *Hear Res.* **117** (1–2): 140–148.
7 Furuta H, Mori N, Sato C, Hoshikawa H, Sakai S, Iwakura S, *et al.* (1994). Mineralocorticoid type I receptor in the rat cochlea: mRNA identification by polymerase chain reaction (PCR) and in situ hybridization. *Hear Res.* **78** (2): 175–180.
8 Furuta H, Mori N, Luo L, Ryan AF (1995). Detection of mRNA encoding guanylate cyclase A/atrial natriuretic peptide receptor in the rat cochlea by competitive polymerase chain reaction and in situ hybridization. *Hear Res.* **92** (1–2): 78–84.
9 Horisberger JD and Rossier BC (1992). Aldosterone regulation of gene transcription leading to control of ion transport. *Hypertension* **19** (3): 221–227.
10 Jard S (1983). Vasopressin isoreceptors in mammals: relation to cyclic AMP-dependent and cyclic-AMP independent transduction mechanisms. *Curr Top Membr Trans.* **18**: 255–285.
11 Koch T, Gloddek B and Gutzke S (1992). Binding sites of atrial natriuretic peptide (ANP) in the mammalian cochlea and stimulation of cyclic GMP synthesis. *Hear Res.* **63** (1–2): 197–202.
12 Lamprecht J and Meyer zum Gottesberge AM (1988). The presence and localization of receptors for atrial natriuretic peptide in the inner ear of the guinea pig. *Arch Otorhinolaryngol.* **245** (5): 300–301.
13 Marcus DC and Chiba T (1999). K+ and Na+ absorption by outer sulcus epithelial cells. *Hear Res.* **134** (1–2): 48–56.
14 Meyer zum Gottesberge A and Lamprecht J (1989). Localization of the atrial natriuretic peptide binding sites in the inner ear tissue--possibly an additional regulating system. *Acta Otolaryngol Suppl.* **468**: 53–57.
15 Meyer zum Gottesberge A, Schleicher A, Drummer C, Gerzer R (1995). The volume protective natriuretic peptide system in the inner ear. Comparison between vestibular and cochlear compartments. *Acta Otolaryngol Suppl.* **520**: 170–173.
16 Potter LR, Abbey-Hosch S and Dickey DM (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev.* **27** (1): 47–72.
17 Seebacher T, Beitz E, Kumagami H, Wild K, Ruppersberg JP, Schultz JE (1999). Expression of membrane-bound and guanylyl cyclases in the rat inner ear. *Hear Res.* **127** (1–2): 95–102.
18 Sterkers O, Ferrary E and Amiel C (1984). Inter- and intracompartimental osmotic gradients within the rat cochlea. *Am J Physiol.* **247** (4 Pt2): F602–F606.
19 Sterkers O, Ferrary E and Amiel C (1988). Production of inner ear fluids. *Physio Rev.* **68**(4): 1083–1128.
20 Tran Ba Huy P and Lecain E (2002). Contribution to the study of endolymph homeostasis. *Bulletin de l Academie Nationale de Medecine.* **186**(7): 1269–1286; *discussion* 1286–1288.
21 Yan L, Yuidi T and Qingjie X (2007). The expression of endothelin type A and B receptors in the lateral wall of the mouse cochlea. *Cell Mol Biol Lett.* **12** (4): 595–603.
22 Yoon YJ and Hellstrom S (1992). Immunohistochemical localization of alpha-atrial natriuretic polypeptide in the rat cochlea. *Acta Otolaryngol.* **112**(4): 604–610.