ORIGINAL ARTICLE

The expression of atrial natriuretic peptide receptor in the mouse inner ear labyrinth

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, P. R. China
2 West China Laboratory of Molecular Genetics, Sichuan University, P. R. China
3 Department of Anesthesiology and Critical Care Medicine, West China Hospital of Sichuan University, P. R. China

Correspondence to: Yuedi Tang, MD., PhD.
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: 2009-09-21 Accepted: 2010-01-25 Published online: 2010-02-16

Key words: **atrial natriuretic peptide receptor; inner ear; stria vascularis; vestibula; real-time quantitative reverse transcription-polymerase chain reaction**

Neuroendocrinol Lett 2010; **31**(1):126–130 **PMID:** 20150880 NEL310110A18

Abstract

OBJECTIVE: To compare the relative expression levels of NPR-A mRNA in the stria vascularis (StV), nonstrial tissue of the cochlear lateral wall (NSt) and vestibula in the mouse inner ear to determine the potential contribution of ANP signaling in different parts to inner ear fluid homeostasis.

SETTING: Atrial natriuretic peptide (ANP) is a cardiac hormone known to be involved in the regulation of body fluid homeostasis. It is assumed that ANP might also participate in the regulation of inner ear fluid dynamics. ANP selectively binds with atrial natriuretic peptide receptor (NPR-A) to exert its physiological function. We have previously shown the presence of NPR-A transcripts in the mouse stria vascularis as well as in the nonstrial tissue of the cochlear lateral wall and vestibular organ by polymerase-chain reaction.

METHODS: Total RNA of samples of stria vascularis, nonstrial tissue of the cochlear lateral wall and vestibular organ tissue from ears of 10 adult mice was isolated, amplified by the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using consensus primers flanking a region of 127 bp at the target sequences. The brain of mice known to contain NPR-A was used as a positive control. The one-way analysis of variance, ANOVA and Student-Newman-Keuls method were performed to analyze the data.

RESULTS: NPR-A mRNA expression was found in tissue samples of all the three areas. The mRNA expression level of NPR-A in the StV was higher than that in the NSt and vestibula ($p<0.05$). The difference of NPR-A mRNA expression level in the NSt and vestibula was not statistically significant ($p>0.05$).

CONCLUSIONS: Our results suggest that the StV may be the most important place for ANP to regulate endolymph fluid balance via NPR-A.

Abbreviations :

ANP	- atrial natriuretic peptide
NPR-A	- atrial natriuretic peptide receptor
StV	- stria vascularis
NSt	- nonstrial tissue of the cochlear lateral wall
RT-PCR	- reverse transcription-polymerase chain reaction
GAPDH	- glyceraldehyde-3-phosphate dehydrogenase
Ct	- cycle threshold
RQ	- relative quantity
SD	- standard deviation
NPR-C	- natriuretic peptide receptor-C
cGMP	- cyclic guanosine monophosphate
BNP	- brain natriuretic peptide receptor

INTRODUCTION

Inner ear which contains osseous labyrinth and membranous labyrinth is an organ responsible for auditory and balance function. Within the bony labyrinth is the membranous labyrinth containing endolymph and the sense organs of hearing and balance (Richard & Mark 2003). Fluid homeostasis expressed by ionic, osmotic and metabolic balances in the endolymphatic system is essential for the performance of inner ear physiological function. Once such balance is disrupted, membrane displacement or abnormal functions of the inner ear can occur, such as endolymphatic hydrops, a condition in which the endolymphatic space of the inner ear is distended secondary to a presumed excess of fluid volume or pressure, or even hearing loss (Guo *et al.* 1994; Juhn 1984; Sterkers *et al.* 1984). It is necessary to clarify regulatory mechanisms of endolymph fluid homeostasis. One of the mechanisms involves in hormones, such as adrenal hormones, aldosterone, atrial natriuretic peptide (ANP), vasopressin and endothelin to regulate electrolyte composition or fluid volume of endolymph (Fujimura *et al.* 1998; Gu *et al.* 2006; Lohuis *et al.* 2000; Luo *et al.* 2007; Suzuki *et al.* 1998; Trune & Kempton 2001; Xu *et al.* 2007). Among these hormones is ANP, which was firstly discovered by DeBold AJ in 1983 (Baines *et al.* 1983). This peptide can regulate blood volume and blood pressure by mediating vasodilation and natriuresis (Potter *et al.* 2006). It regulates ocular and cerebrospinal fluid composition and/or pressure (Mantyh *et al.* 1986). It was postulated that ANP might also regulate fluid homeostasis of endolymph.

ANP binds to atrial natriuretic peptide receptor (NPR-A) with high affinity to exert its biological role by increasing guanylyl cyclase activity (Potter *et al.* 2006). Detection of NPR-A mRNA expression in the inner ear is necessary to elucidate the mechanism of the regulation of endolymph fluid balance in the inner ear by ANP. Several studies have identified the existence of ANP receptors in the inner ear of guinea pig and rat, and have demonstrated them in the stria vascularis (StV), nonstrial tissue of the cochlear lateral wall (NSt) and vestibula (Koch *et al.* 1992; Lamprecht & Meyer 1988; Meyer & Lamprecht 1989; Meyer *et al.* 1995; Seebacher *et al.* 1999). We have previously detected the transcripts of NPR-A in the mouse inner ear by polymerase-chain reaction (PCR) (Long *et al.* 2008). In this study, we compared the relative expression levels of NPR-A mRNA within the secretory areas in the adult mouse inner ear using real-time quantitative reverse transcription-PCR (RT-PCR) to further determine the potential contribution of ANP signaling in different regions of the inner ear to the regulation of endolymphatic fluid homeostasis.

MATERIALS AND METHODS

Animals

The Kunming mouse which is an outbreed strain of laboratory animal widely utilized in related pharmaceutical and genetic studies in China was used in this study. Ten healthy adult Kunming mice (30 days old) were provided by the Animal Care and Use Committee at the West China Medical School of Sichuan University. The study 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, StV, NSt and tissue of vestibula in the inner ear were dissected at 4 °C as described previously (Luo *et al.* 2007). The brain of the adult mice was used as positive control.

RNA isolation and reverse transcription

Total RNA from the tissues of the StV, NSt, vestibula and brain of the adult mice was isolated using Trizol reagent (MRC USA). The RNA was reverse transcribed into cDNA using the Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas Inc.) with addition of random hexamer primers. A total of 5 µl of purified RNA, 1 µl M-MLV reverse transcriptase (200 U), 1 µl of random hexamer primer, 4 µl of 5× reaction buffer, 2 µl of 1× hexamer (Roche), 2 µl of dNTP mix (10 mM each), and 6 µl of RNAse-free water were used for cDNA synthesis. The reverse transcriptase was inactivated at 70 °C for 10 min after incubation. The cDNAs were stored at –20 °C until further analysis.

Primers

The primer pairs for NPR-A cDNA PCR amplification were designed according to the NCBI Genebank of mice (Accession number: ENSMUSP00000029540). The primers were synthesized by the Shanghai SAGON. The primer sequences were as follows: forward primer 5'-CGACGGGCTCCTGCTCTAT-3' and reverse primer 5'-CAGGTATCCTGTCACACCTTG-3' (127 bp). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was detected as the inner control. The primers for mouse GAPDH were as follows: forward primer 5'-CCTCAAGATTGTCAGCAAT-3' and reverse primer 5'-CCATCCACAGTCTTCTGGGT-3'

(141 bp). cDNA templates were treated with DNase I before PCR amplification. Positive control amplification was performed using cDNA from the mouse brain as templates. PCR without templates was performed as a negative control.

Real-time quantitative PCR

Real-time PCR was used to determine the gene expression profiles of NPR-A. cDNAs were amplified using FTC-2000 (FUNGLYN, CANADA). Each analysis was performed in a total volume of 25 µl reaction mixture containing 5 µl cDNA sample, 2 µl SYBR GREEN I, and 2 µl gene-specific forward and reverse primers (10 µM each). GAPDH genes were included to normalize the data. Amplications were 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 54 °C, extension for 30 sec at 72 °C and for 20 sec at 80 °C. The amplified PCR products were routinely assessed by horizontal electrophoresis in 2% agarose gels containing 1 µl/ml of ethidium bromide.

The cycle threshold (Ct) number (the number of PCR amplification cycles required to reach fluorescent intensity above the threshold) was determined for developmental time point of gene analyzed. Using serial dilutions of the test sample cDNA, the standard curve was generated on the basis of the linear relationship of existing Ct and the logarithm of the copy number. The slope of the curve was shown to be –3.55, and a strong linear relationship was demonstrated (R^2=1.00; Figure 1).

Analysis

Relative gene expression data was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Samples were normalized internally using the Ct number of the housekeeping gene GAPDH, as follows: ΔCt (sample) = Ct (sample) – Ct (GAPDH). The Ct of NPR-A cDNA from brain was set to a relative quantity (RQ) value of 1 using the ΔΔCt, calculated as follows: ΔΔCt (sample) = ΔCt (sample) – ΔCt (brain), and RQ = $2^{-\Delta\Delta Ct}$. The data is reported as means ± standard deviation (SD). The one-way analysis of variance, ANOVA and Student-Newman-Keuls method were performed to analyze the data. $p<0.05$ was considered to be statistically significant.

RESULTS

The results of the analysis of NPR-A mRNA expression from StV, NSt and vestibula were shown in Figure 2. The expression level of NPR-A mRNA in the StV was predominant and higher than that in the NSt and vestibula ($p<0.05$). The difference of NPR-A mRNA expression level in the NSt and vestibula was not statistically significant ($p>0.05$).

DISCUSSION

In this study, real-time quantitative RT-PCR method was used to determine NPR-A mRNA expression in StV, NSt and vestibula of adult mouse inner ear. PCR is a rapid and powerful technique for *in vitro* amplification of DNA. Being specific and sensitive, real-time quantitative RT-PCR has been used widely to measure mRNA expression.

The homeostasis of inner ear endolymph is important for the maintenance of the normal physiological function of this organ. Regulation of endolymph production by hormones is one of the mechanisms maintaining endolymph fluid balance. ANP, a classical hormone

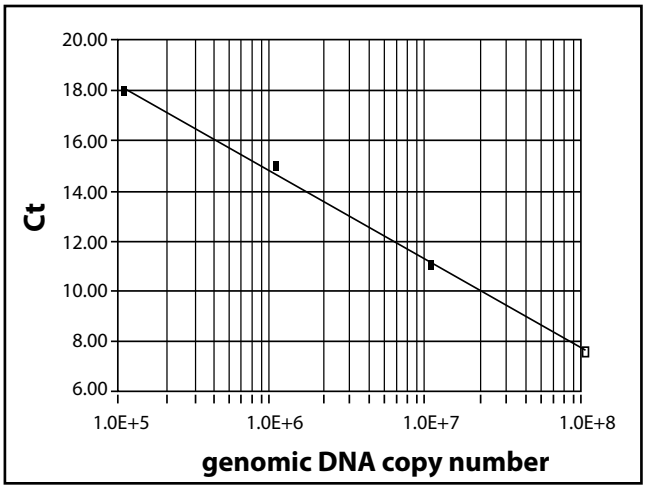

Fig.1. The standard curve for the GAPDH set generated from Ct values and plotted against the input standard genomic DNA molecules. The standard curve ranged from 105 copies to 108 copies. Using serial dilutions of the standard cDNA, the standard curve was generated on the basis of linear relationship between the existing Ct and the logarithm of the copy number.

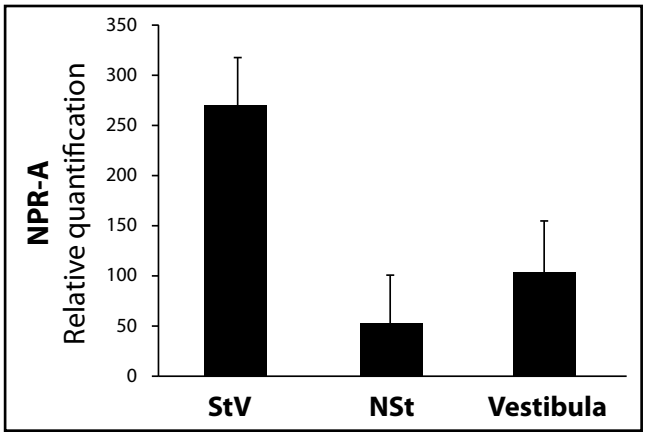

Fig. 2. The expression of NPR-A mRNA in mice inner ear by real-time quantitative RT-PCR. The quantification of NPR-A gene expression in the StV (n=6), NSt (n=6) and vestibula (n=5) relative to the brain (n=1). The expression of NPR-A mRNA was normalized to the expression of GAPDH mRNA in the inner ear and brain, separately. Significantly different expression ($p<0.05$) and non-significantly different expression ($p>0.05$) were expressed as the average ± SD.

regulating fluid volume and pressure was observed in the inner ear (Chen *et al.* 1994; Meyer *et al.* 1995). It regulates Na^+ concentration and pressure of endolymph in an autocrine or paracrine manner (Bartoli *et al.* 1989; Chen 1993). It has also been shown to regulate fluid balance of endolymph as a circulation hormone (Borghi *et al.* 2006). In addition, ANP participates in blood flow regulation in the inner ear (Lin & Qiu 2005). Hormone receptors on the cell surface have a high selectivity for a specific hormone. When the hormone binds to a specific receptor, the receptor-hormone complex initiates cascade reactions that change the biological function of the cell. ANP can bind to NPR-A or natriuretic peptide receptor-C (NPR-C) to induce different physiological responses in cells from different tissues (Potter *et al.* 2006). Thus, the identification of a specific ANP receptor is crucial to understand the regulatory mechanism of ANP involving in the inner ear fluid homeostasis. NPR-C clears natriuretic peptides from the circulation through receptor-mediated internalization and degradation, while NPR-A stimulates their physiological functions through specific signal pathways in tissues. It can increase capillary permeability, vasodilation, and natriuresis by directly modulate sodium channels and transporters, activate protein kinases, or stimulate cyclic guanosine monophosphate (cGMP) -hydrolyzing phosphodiesterase$_2$ (Potter *et al.* 2006). Thus, NPR-A is the candidate receptor for ANP function in the regulation of endolymph fluid homeostasis.

NPR-A is a natriuretic peptide binding protein. It consists of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain, which can catalyze guanylyl cyclase. By activating guanylate cyclase, one of the intracellular second messenger cAMP increases and initiates the cascade reactions needed in biological functions of cells (Potter *et al.* 2006). Both ANP and brain natriuretic peptide (BNP) can bind with NPR-A. BNP is not existed in the inner ear, thus NPR-A can only be activated by ANP (Suzuki *et al.* 1998). ANP mRNA was detected in StV of rat (Cao *et al.* 2007; Zhang *et al.* 2008). Although the exact mechanism of action has not been elucidated, the coexistence of ANP mRNA and NPR-A mRNA expression in the StV of inner ear suggested that ANP may regulate fluid and electrolyte balance in the endolymphatic system through NPR-A in a paracrine/autocrine manner.

The StV, NSt in cochlea duct and the vestibula containing utricle and saccule are structures believed to be important for endolymph generation. The overproduction of endolymph in the secretory areas of inner ear, such as StV and dark cell epithelium in the vestibular organs is one of the causes of Meniere's disease. Our previous studies have demonstrated the presence of NPR-A transcripts in StV, NSt in cochlea duct and the vestibula in the inner ear by a sensitive RT-PCR analysis (Long *et al.* 2008). The results of the present study by real-time quantitative RT-PCR were in agreement with previous studies (Krause *et al.* 1997; Long *et al.* 2008; Seebacher *et al.* 1999), suggesting that StV is the main place of NPR-A synthesis among the three secretory areas for endolymph fluid regulation. StV is full of microvascula. NPR-A may also be involved in blood flow regulation. NPR-A in the vestibula may play a less important role than that in the StV. NPR-A in the NSt might also regulate endolymgh fluid balance. The actual mechanism of endolymph fluid balance regulation by NPR-A needs to be elucidated by further investigation, such as electrophysiological research using patch-clamp technique.

The presence and localization of ANP receptors was reported to be in the inner ear of the guinea pig (Lamprecht & Meyer 1988). Since then, many studies have shown the existence of natriuretic peptide receptors in the inner ear and the possible biological roles have also been reported (Furuta *et al.* 1998; Furuta *et al.* 1995; Koch *et al.* 1992; Meyer & Lamprecht 1989; Meyer *et al.* 1995; Seebacher *et al.* 1999). Results of further study by Koch *et al.* showed an increase of cGMP in the endolymph stimulated by local infusion of ANP into the inner ear, suggesting indirectly the existence of NPR-A within StV (Koch *et al.* 1992). Our study is a complement of previous research.

In conclusion, we demonstrated that NPR-A mRNA was expressed in StV, NSt and vestibula in the adult mouse inner ear using real-time quantitative RT-PCR. Further data analysis suggested that gene expression of NPR-A in the StV was higher than that in the NSt and vestibula. Among the three areas under investigation, StV may be the most important part in terms of ANP-mediated physiological role through NPR-A. As StV is an important structure of endolymph fluid production and is full of microvascula, NPR-A in the StV is likely involved in the regulation of endolymph fluid balance and blood flow. NPR-A may be activated by ANP in a paracrine/autocrine manner to stimulate cGMP production. Further investigations are needed to elucidate the direct interaction of ANP and NPR-A in secretory areas of the inner ear, especially in StV, and their physiological role in the regulation of endolymph fluid homeostasis.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Yanfang Chen and Mr. Faqiang Zhang for their technical assistance. This project was supported by National Natural Science Foundation of China (NSFC 30271408 and 30872844 to Dr. YD Tang).

REFERENCES

1 Baines AD, DeBold AJ and Sonnenberg H. (1983). Natriuretic effect of atrial extract on isolated perfused rat kidney. Can J Physiol Pharmacol. **61**(12): 1462–1466.

2 Bartoli E, Satta A, Melis F, Caria MA, Masala W, Vargiu G, *et al.* (1989). Volume receptors in guinea pig labyrinth: relevance with respect to ADH and Na control. Am J Physiol. **257**(3 Pt 2): F341–346.

3 Borghi C, Modugno GC, Brandolini C, Pirodda A. (2006). Is there a role for atrial peptides in the labyrinthine "disease"? Med Hypotheses. **66**(6): 1188–1190.
4 Cao Z, Gao X, and Qiu J. (2007). Expression of atrial natriuretic peptide (ANP) and its receptor in the rat's cochlea of different age groups. Chin J Otol. **5**(4): 439–441.
5 Chen H. (1993). Immunohistochemical distribution and fine localization of atrial natriuretic peptides in the stria vascularis of normal guinea pigs. Chin J Otorhinolaryngol Head Neck Surg. **28**(6): 339–341, 380–381.
6 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.
7 Fujimura T, Furukawa H, Doi Y, Fujimoto S. (1998). The significance of endothelin for generation of endocochlear potential. J Cardiovasc Pharmacol. **31**(suppl.1): S376–377.
8 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.
9 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.
10 Gu FM, Han HL and Zhang LS. (2006). Effects of vasopressin on gene expression in rat inner ear. Hear Res. 222(1–2): 70–78.
11 Guo Y, Wu Y, Chen W, Lin J. (1994). Endotoxic damage to the stria vascularis: the pathogenesis of sensorineural hearing loss secondary to otitis media? J Laryngol Otol. **108**(4): 310–313.
12 Juhn SK. (1984). Inner ear fluids dynamics and endolymphatic hydrops. Acta Otolaryngol Suppl. **406**: 26–30.
13 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.
14 Krause G, Meyer zum Gottesberge AM, Wolfram G, Gerzer R. (1997). Transcripts encoding three types of guanylyl-cyclase-coupled trans-membrane receptors in inner ear tissues of guinea pigs. Hear Res. **110**(1–2): 95–106.
15 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.
16 Lin Y and Qiu JH. (2005). Distribution of atrial natriuretic peptides in cochlear microvascular of guinea pigs. J Fourth Mil Med Univ. **26**(7): 581–583.
17 Livak KJ and Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Meth (Duluth). **25**(4): 402–408.
18 Lohuis PJ, Borjesson PK, Klis SF, Smoorenburg GF. (2000). The rat cochlea in the absence of circulating adrenal hormones: an electrophysiological and morphological study. Hear Res. **143**(1–2): 189–196.
19 Long L, Tang Y, Xia Q, Xia Z, Liu J. (2008). Detection of atrial natriuretic peptide receptor in the labyrinth of the mouse inner ear. Neuroendocri Lett. **29**(4): 577–580.
20 Luo Y, Tang Y, Liu J, Chen Y, Xia Q. (2007). Endothelin-1 inhibits outward potassium currents in mouse outer sulcus cells. Cell Mol Biol. **53** Suppl: OL981–988.
21 Luo Y, Tang Y, Xia Q, Liu J. (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 Mantyh CR, Kruger L, Brecha NC, Mantyh PW. (1986). Localization of specific binding sites for atrial natriuretic factor in peripheral tissues of the guinea pig, rat, and human. Hypertension. **8**(8): 712–721.
23 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.
24 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.
25 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.
26 Richard RG and Mark RG. (2003). Anatomy of Auditory and Vestibular Systems. In: James BS Jr, editor. Ballenger's Otorhinolaryngology Head and Neck Surgery. 16th ed. Chicago: BC Decker Press. p. 9.
27 Seebacher T, Beitz E, Kumagam i 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.
28 Sterkers O, Ferrary E and Amiel C. (1984). Inter- and intracompartimental osmotic gradients within the rat cochlea. Am J Physiol. **247**: F602–F606.
29 Suzuki M, Kitano H, Kitanishi T, Yazawa Y, Kitajima K, Takeda T, *et al.* (1998). RT-PCR analysis of mRNA expression of natriuretic peptide family and their receptors in rat inner ear. Brain Res Mol Brain Res. **55**(1): 165–168.
30 Trune DR and Kempton JB. (2001). Aldosterone and prednisolone control of cochlear function in MRL/MpJ-Fas(lpr) autoimmune mice. Hear Res. **155**(1–2): 9–20.
31 Xu DY, Tang YD, Liu SX, Liu J. (2007). Distribution and significance of endothelin 1 in guinea pig cochlear lateral wall. J Laryngol Otol. **121**(8): 721–724.
32 Zhang P, Chen J, Xue T, Cao Z, Qiu J. (2008). Expression of atrial natriuretic peptides in marginal cells of the rat stria vascularis in primary culture. Chin J Otol. **6**(3): 325–330.