Natriuretic Peptide Receptors in the Human Endolymphatic Sac

John L. Dornhoffer, MD; Christopher Danner, MD; Shulin Li, PhD

Objective: To examine human endolymphatic sac (ELS) tissue for atrial natriuretic peptide (ANP) receptor subtypes A, B, and C.

Design: Pilot study.

Methods: Immunohistochemical analysis of human ELS tissue specimens. The ANP receptors were characterized using the peroxidase/antiperoxidase method and polyclonal antibodies directed against each receptor subtype. The identity of the stain regarding receptor subclass was masked from the observer. Human kidney tissue known to contain all 3 receptor subtypes was used as a control. Presence of the receptor subclasses was confirmed using reverse transcriptase–polymerase chain reaction (RT-PCR) techniques.

Subjects: Samples of ELS tissue were obtained at autopsy from 3 fresh cadaver specimens (6 ears) and as surgical specimens from 3 patients (1 for immunohistochemical analysis and 2 for RT-PCR) undergoing acoustic neuroma resection using the translabyrinthine approach.

Results: The ANP type B receptors demonstrated moderate to strong reactivity in all 7 specimens, and mild to moderate staining to the ANP type C receptor was also noted. No appreciable reactivity to the ANP type A receptor was detected using immunohistochemical techniques. All 3 receptor subclasses were detected using RT-PCR.

Conclusions: The ANP receptors are found within the human ELS, with a predominance of ANP type B based on the intensity of staining. The ANPs may be involved in fluid homeostasis in the inner ear. Based on these findings, C-type natriuretic peptide may be a more effective peptide within the human ELS for fluid regulation because its binding affinity is virtually exclusive for the ANP type B receptor.


FLUID HOMEOSTASIS in the endolymphatic system of the inner ear is essential for normal hair cell function, but the mechanisms involved with its maintenance, to date, are poorly understood. It is believed that endolymph is produced by active transport of electrolytes in the stria vascularis of the cochlea and the dark cell epithelium of the vestibular organ and that it is subsequently reabsorbed by the endolymphatic sac (ELS). The ELS epithelium synthesizes and secretes certain complex molecules, including hyaluronan, into the ELS lumen and is postulated to be involved in the sensitive regulation of endolymphatic volume and pressure. Thus, dysfunction of the ELS is thought to be the underlying cause of the endolymphatic hydrops associated with Meniere’s disease, a disease with no accepted cure.

Research has suggested a possible role of atrial natriuretic peptides (ANPs) in the regulation of inner ear fluid. The ANPs are a group of hormones originally identified in mammalian atrial cardiocytes in the early 1980s. They are known to exert a smooth muscle effect, with vasodilation, diuretic, and natriuretic activities in the kidney. The natriuretic peptide system comprises at least 3 known peptides—ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)—and 3 receptor subtypes. Two of the receptors, ANP type A (ANP-A) and ANP type B (ANP-B), represent the bioactive receptors, whereas ANP type C (ANP-C) is biologically inactive and serves as a clearance receptor. Natriuretic peptides have been located in other extracardiac sites, such as the adrenal cortex, lung parenchyma, gallbladder, ciliary body of the eye, and choroid plexus of the brain.

Investigators have demonstrated the presence of ANP receptors in the inner ear of the guinea pig, with ANP-like immunoreactivity demonstrated in the co-
**MATERIALS AND METHODS**

**SPECIMEN COLLECTION**

Human ELSs were obtained from 3 fresh cadaver specimens (6 ears) and from 3 patients undergoing a translabyrinthine approach for acoustic neuroma resection (1 for immunohistochemical analysis and 2 for RT-PCR). Patients gave informed consent for the study, which had received approval by the institutional review board at the University of Arkansas for Medical Sciences, Little Rock. The entire ELS was removed en bloc with a portion of posterior fossa bone and otic capsule. Control samples consisted of human kidney tissue known to contain all 3 receptor subtypes.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Specimens were placed in 10% neutral buffered formaldehyde, followed by decalcification in formic acid. After completion of decalcification (10-14 days), the samples were embedded in paraffin. The specimens were then serially sectioned (4-5 µm) parallel to the long axis of the ELS, fixed, and mounted on appropriately labeled slides. The sections were hydrated in the usual manner, terminating in phosphate-buffered saline solution.

Tissue sections were incubated in 3% hydrogen peroxide and phosphate-buffered saline solution to block endogenous peroxidase activity. Polyclonal antibodies (rabbit and anti-rat) directed against the ANP-A, ANP-B, and ANP-C receptors were obtained from David Garbers, MD, Southwestern Medical Center, University of Texas, Dallas. Each individual clone was incubated with the specimens at concentrations of 1:100 and 1:1000. The 1:100 dilution was used for analysis because it gave the best staining with the least background activity. Steam was used to increase antigen retrieval in half of the specimens, but no significant difference was seen with this technique. The specimens were then incubated with diamobenzidine–hydrogen peroxide, which reacts with the peroxidase-antibody complex to produce a red-brown stain. The slides were counterstained with Mayer hematoxylin to visualize the nuclei and were studied with conventional light microscopy.

Technique controls were performed for each specimen using the same method but omitting the antibody step. Representative slides from each specimen set were also stained with hematoxylin-eosin for additional light microscopic study. For each sample, the identity of the stain regarding receptor subclass was masked from the observer to minimize observer bias in quantifying relative staining intensity compared with background staining.

**RT-PCR ANALYSIS**

To confirm the presence of the subclasses of ANP receptor genes in human ELS specimens, RT-PCR analysis was performed on 2 ELS specimens obtained at surgery during resection of acoustic neuromas using the translabyrinthine approach. Each sample was placed in liquid nitrogen. Human kidney tissue samples were used as a positive control. To isolate RNA for RT-PCR, the harvested tissues were retrieved from the liquid nitrogen and homogenized using a Polytron homogenizer (Brinkmann Instruments, Inc, Westbury, NY). Reagent (TRizol; Gibco, Grand Island, NY) was added at a concentration of 1 mL per 100 to 200 mg of tissue, and the material was spun at 14,000 rpm for 10 minutes. The supernatant was transferred to a new tube, mixed with 200 µL of chloroform, and spun. The supernatant was then isolated and mixed with 500 µL of 2-propanol. The material was cooled to -20°C for 30 minutes and then spun at 14,000 rpm for 20 minutes. The pellet was washed twice with 75% ethanol and then dissolved in 20 to 100 µL of DEPC-H₂O. Optical density was measured at 260 nm to obtain the RNA concentration. The RNA was then purified by DNeasy treatment. A total of 30 µg of RNA in 51 µL of DEPC-H₂O was mixed with 10×DNase I buffer (6 µL) and 1 U/µL of DNase I (3 µL) (Clontech, Palo Alto, Calif) and incubated at 37°C for 30 minutes. To this was added 10×termination buffer (6 µL), followed by 66 µL of phenol-chloroform (1:1). The solution was spun at 14,000 rpm for 10 minutes. The supernatant was isolated, mixed with 66 µL of chloroform, and spun at 14,000 rpm for 10 minutes. The toluene layer was transferred and mixed with a one-tenth volume of 3M sodium acetate and 2 volumes of ethanol and cooled for 30 minutes on ice. The mixture was spun, and the pellet was washed and then dissolved in DEPC-H₂O. This yielded 10 to 20 µg of purified RNA.

Reverse transcriptase–polymerase chain reaction was performed using a commercially available reverse transcription procedure (First-Strand cDNA Synthesis; Amersham Pharmacia Biotech, Piscataway, NJ). A total of 5 µg of RNA in 33 µL of DEPC-H₂O was heated at 65°C for 10 minutes and then chilled on ice for 2 minutes. The RNA solution was transferred to a tube containing First-Strand Reaction Mix Beads (Amersham Pharmacia Biotech), 2 µL of random primer was added, and the contents were mixed by gentle vortex and then incubated at 37°C for 60 minutes. The total volume of first-strand complementary DNA was 33 µL. Using complementary DNA as a template, the primers of the ANP-A, ANP-B, and ANP-C receptors were designed as follows:

- **ANP-A receptor** (length: 500 base pair [bp]): sense, 5'-AACCTGACGGTACCGCTTGATC-3'; antisense, 5'-TGCCTCTTTCTGTCAGA-3';
- **ANP-B receptor** (length: 454 bp): sense, 5'-ATGGCCGTGACATCATCTTCT-3'; antisense, 5'-ACTCAACCAGTGGGAGCA-3';
- **ANP-C receptor** (length: 579 bp): sense, 5'-ACGATGGCGTCTCTGCTGATG-3'; antisense, 5'-CCTTGTGCCCGCTGTAGT-3';
- Reduced glyceraldehyde-phosphate dehydrogenase (length: 354 bp): sense, 5'-AGGCTGAGATGGGAAG-3'; antisense, 5'-AGTACTCGGAAATGGC-3';

The PCR conditions were as follows: denaturation at 94°C for 2 minutes, followed by 37 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute. The PCR mixture was incubated for 10 minutes at 72°C after the amplification cycles and then stored at 4°C or used immediately for electrophoresis.

chlea, the secretary epithelium of the vestibular organ, and the ELS. The results of these animal studies suggest that the inner ear may have paracrine or autocrine activity for the regulation of labyrinthine fluids and electrolytes. The purpose of this study was to characterize specific ANP receptor subtypes within the human ELS.
using immunohistochemical techniques, with confirmation by reverse transcriptase–polymerase chain reaction (RT-PCR).

**RESULTS**

The entire ELS was identified in all cases. Moderate-to-strong staining in the epithelium of the ELS was demonstrated to the ANP-B receptor in all 7 samples. Staining of the ANP-B receptor was present throughout the ELS but had the highest concentration in the rugose portion (Figure 1B). All samples showed slight-to-moderate staining to the ANP-C receptor in the epithelium, with a distribution similar to that of the ANP-B receptor (Figure 1C). No appreciable staining was noted for the ANP-A receptor subclass (Figure 1A). Control specimens demonstrated positive staining for all 3 receptor subtypes in the kidney tissue, and the technique controls appropriately had negative findings.

The results of RT-PCR confirmed that all 3 receptors could be detected in the human ELS sample (see Figure 2 for RT-PCR of human ANP-B).

**COMMENT**

Although the ELS was once thought to represent a vestigial organ of embryogenesis, it is now known to have an essential role in inner ear homeostasis. Systemic administration of hypertonic agents, such as glycerol, has been shown to provoke a transient improvement in hearing in patients with the endolymphatic hydrops associated with Meniere’s disease through mechanisms that were initially unclear. More recent research in animals has demonstrated morphologic changes in the ELS associated with the systemic administration of glycerol, including increased metabolic activity, with the secretion of osmotically active glycoproteins into the lumen of the sac and subsequent lysis and breakdown of these substances. It has thus been suggested that the ELS affects fluid homeostasis in the endolymph system through modulation of the osmotic milieu of this space, with secretion of osmotically active substances into its lumen. The exact mechanism through which the ELS regulates this system is unknown, but it is postulated to involve a locally effective paracrine system, possibly involving the cochlear duct or stria vascularis. Dysfunction of the ELS or breakdown of this system is believed to result in the endolymphatic hydrops of Meniere’s disease.

Results of recent studies have shown that ANPs and ANP-like receptors exist in the inner ear. Their involvement with fluid homeostasis in other organ systems indicates that they could be involved in normal inner ear fluid regulation. Our current understanding of natriuretic peptides, their specific receptors, and their natriuretic, diuretic, and vasodilator effects are the result of numerous studies already performed on cardiac and renal tissue. Myocardiocytes seem to be the major site of synthesis and secretion for ANP; however, immunohistochemical activity of ANP has been observed in a variety of extracardiac tissues, such as the brain, kidney, adrenal medulla, salivary glands, ciliary process of the eye, and anterior pituitary gland. Brain natriuretic peptide and CNP, first isolated from porcine brain tissue, have also been located in other sites, including the heart (BNP), kidney (CNP), and gastrointestinal tract (CNP).

Three types of natriuretic peptide receptors have been identified and characterized. Two of these, ANP-A and ANP-B, represent the bioactive receptors, and they consist of an extracellular domain for natriuretic peptide binding, a single transmembrane domain, a single adenosine triphosphate–binding domain for natriuretic peptide binding, and a guanylyl cyclase moiety. By activating guanylate cyclase, intracellular cyclic guanosine monophosphate concentrations increase and serve as a second messenger at the cellular level in various target tissues. Recently, a third receptor, ANP-C, has been identified. The ANP-C receptor, which is not coupled to gua-
narylate cyclase, is thought to be biologically silent and to serve as a specific clearance binding site for natriuretic peptides. 25 The 3 natriuretic peptides exhibit different binding affinities to the 3 receptor subtypes.26 The ANP-A receptors preferentially bind ANP over BNP, with little reactivity for CNP. The ANP-B receptors seem to be highly selective for CNP, with an affinity that is 50- to 500-fold higher than for ANP or BNP. The ANP-C receptors bind all 3 natriuretic peptides, but with a rank order of ANP>BNP>CNP.

Based on the knowledge that natriuretic peptides are located within extracardiac tissues known to play a role in the maintenance of fluid/electrolyte homeostasis (ie, ciliary process of the eye and choroid plexus of the cerebral ventricles), it has been postulated that they may also be present in the inner ear and have similar activity. In the late 1980s, Lamprecht and Meyer zum Got-tesberge and their colleagues3,4 demonstrated the presence and localization of ANP receptors within the inner ear of the guinea pig. Using autoradiographic techniques with radiolabeled ANP/cardiodilantin, receptors were localized to the stria vascularis, the organ of Corti, spiral ganglion cells, and the epithelial layer of the pars rugosa of the ELS. Results of additional studies5,27 have also confirmed the presence of peptide receptors in the inner ear. Results of a study by Rachel et al28 showed that infusion of ANP directly into the inner ear of guinea pigs contributes to the regulation of vestibular blood flow. Results of a more recent study29 suggest that epithelial cells of the ELS contain an endogenous hormone, tentatively named “saccin,” that exerts a strong natriure- sis. In 1997, Krause et al30 identified the presence of ANP-A and ANP-B receptor subtypes within inner ear tissue obtained from guinea pigs using RT-PCR. They also identified a new guanylyl cyclase receptor, GC-C, which is normally present in the intestines. Their PCR analysis did not demonstrate the presence of any clearance receptors (ANP-C).

To our knowledge, this study is the first to identify specific natriuretic peptide receptors within the human ELS. The combination of immunohistochemical and PCR techniques confirmed the presence of all 3 receptor subclasses, with an apparent predominance of the ANP-B receptor based on intensity of staining with immunoperoxidase. The ANP-A receptor seemed to be present based on the sensitive, but nonquantitative, RT-PCR, but the expression of this receptor was apparently low based on the inability to detect it against background staining using the less sensitive immunoperoxidase techniques.

These findings imply that CNP, not ANP, may be a more effective peptide within the human ELS for fluid regulation because its binding affinity is virtually exclu- sive for the ANP-B receptor, which showed the strongest activity.31,32 Earlier studies showed only minimal immunoreactivity when using radiolabeled ANP,4 suggesting that few if any receptors were present within the ELS. This information is consistent with our data because ANP is known to have weak affinity for the ANP-B receptor, the predominant subtype demonstrated in this study.26 C-type natriuretic peptide was first isolated from porcine brain in 1990, and it is the major natriuretic peptide in human cerebrospinal fluid.17,33 Results of previous studies33 have shown that CNP stimulates excretion of sodium and water from the kidney and thereby suggest that it may also play a role in the regulation of water and electrolyte homeostasis within the inner ear.

The findings reported herein lend some credence to the hypothesis that fluid homeostasis in the endolymphatic system could be regulated by a locally effective paracrine system involving the ANP system, analogous to other organ systems in the body. The exact mechanism of action has not been elucidated but intuitively would involve either regulation of fluid production by the stria vascularis or absorption by the ELS.28 It has been suggested that the ELS affects fluid homeostasis in the endolymphatic system through modulation of the osmotic milieu of this space, with the secretion of osmotically active substances into its lumen; however, the mechanism of action is unknown.30 It is conceivable that ANPs could be involved in this mechanism. It is likewise possible that the ANP system could be involved in fluid production by the stria vascularis.28 The natriuretic properties of ANPs in the kidney are well-known, and their association with excretory organs such as the gallbladder and choroid plexus supports this notion. Although ANP receptors have
been demonstrated in the inner ear, their association with fluid transport in the endolympathic system is, at this point, only conjecture. Animal studies are currently ongoing at this institution (Department of Otolaryngology/Head and Neck Surgery, University of Arkansas for Medical Sciences) to investigate these unique peptides and to define their role within the inner ear.

In conclusion, (1) the natriuretic peptide receptors are found within the human ELS, with a predominance of ANP-B based on the intensity of staining; (2) the ANP system may be involved in fluid homeostasis in the human endolympathic system; and (3) CNP, rather than ANP, may be a more effective peptide within the human ELS for fluid regulation because its binding affinity is virtually exclusive for the ANP-B receptor.

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Corresponding author and reprints: John L. Dornhoffer, MD, Department of Otolaryngology/Head and Neck Surgery, University of Arkansas for Medical Sciences, 4301 W Markham, MS 543, Little Rock, AR 72205 (e-mail: DornhofferJohnL@uams.edu).

REFERENCES


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