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Research paper

Effects of vasopressin on gene expression in rat inner ear

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Abstract

Vasopressin regulates water excretion from the kidney by increasing water permeability of the collecting duct as a hormone secreted from the posterior pituitary. A clinical study reported that plasma levels of arginine vasopressin were significantly higher in patients suffering from Meniere's disease. It was histologically confirmed that chronic administration of vasopressin induced endolymphatic hydrops in guinea pigs. However, the mechanism of endolymphatic hydrops induced by vasopressin is still unclear. We use cDNA microarray to study the effects of vasopressin on gene expression profiles in rat inner ear to elucidate the possible mechanism of the induced hydrolabyrinth. Wistar rats were intraperitoneally injected with 50 μg/kg arginine vasopressin once a day for one week. Hydrolabyrinth in rat inner ear induced by administration of vasopressin was detected by HE stain. The bullae were dissected out for total RNA extraction. cDNAs were synthesized by reverse transcription and labeled with Cyanine3 (Cy3) or Cyanine5 (Cy5). The BiostarR-40s cDNA microarray was hybridized with the above cDNAs and the changes of mRNA expression intensity were showed by data analysis. Furthermore, the changes of aquaporins expression level were measured by reverse transcription polymerase chain (RT-PCR). Endolymphatic hydrops were present in rats intraperitoneally injected with vasopressin. 226 known differentially expressed genes were screened out in rat inner ear induced by vasopressin injection. Of the 226 genes, 18 transcripts were increased by 5-fold or more, and 7 transcripts were decreased to 0.2-fold or less. Ten differentially expressed genes were identified that associate with cell signal transduction, 14 differentially expressed genes were identified that relate to ion transport, 7 differentially expressed genes were involved in vesicle-mediated transport, and 2 differentially expressed genes were aquaporin 2 (AQP2) and aquaporin 7 (AQP7). The expression level of AQP2 was significantly higher and AQP7 was significantly lower. These results suggest that there are obvious differences in gene expression profiles in inner ear between vasopressin injected rats and normal control rats. Vasopressin may disturb fluid homeostasis in inner ear by way of signal transduction, ion transport, vesicle-mediated transport and aquaporins. It is likely that up-regulated expression of AQP2 mRNA and down-regulated expression of AQP7 mRNA in the rat inner ear caused by vasopressin induce an increased production and a decreased absorption of endolymph, resulting in endolymphatic hydrops.

Keywords: Vasopressin; Inner ear; Gene expression; Microarray; Aquaporin; Endolymphatic hydrops

1. Introduction

As a hormone secreted from the posterior pituitary, vasopressin regulates water excretion from the kidney by increasing water permeability of the collecting duct. The plasma concentrations of vasopressin were significantly elevated in patients with inner ear disorders caused by endolymphatic hydrops, including Meniere's disease (Takeda et al., 1995). Two cases, twins, affected by congenital nephrogenic diabetes insipidus with a high daily volume of dilute urine excretion and periods of compensatory high levels of antidiuretic hormone (ADH) simultaneously

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developed a fluctuating Meniere-type hearing loss (Comacchio et al., 1992). Clinical evidence suggested a causative role for vasopressin as a stress hormone in patients with Meniere's disease. Many patients with Meniere's disease were reported various stresses before the onset of vertigo (Sawada et al., 1997) which would increase the plasma level of vasopressin (Dugue et al., 1993). Animal experiments indicated that vestibular stimulation increased plasma levels of vasopressin involved in a mechanism of vestibulo-autonomic responses (Horii et al., 2001). In contrast, the mean ADH level for patients with unilateral Meniere's disease was 4.07 pg/ml (SD = 2.82 pg/ml) and for normal control was 3.37 pg/ml (SD = 1.48 pg/ml). The difference in ADH levels was not statistically significant (Lim et al., 2003). There was no statistically significant difference in the plasma vasopressin level between the hydrops models and normal guinea pigs (Kitano et al., 1994). Anyhow, histological experiments have showed that vasopressin caused endolymphatic hydrops after systemic application to guinea pigs, which suggested a causal role for the increased vasopressin levels found in patients suffering from Meniere's disease (Kumagami et al., 1998). The formation of endolymphatic hydrops in guinea pigs might be caused by the high plasma vasopressin concentration as those observed in the acute phase of Meniere's disease (Takeda et al., 2000).

In renal collecting duct, vasopressin binds to vasopressin V2 receptor (V2R), leading to activation of adenylate cyclase (AC) and elevation of cyclic adenosine monophosphate (cAMP), resulting in activation of cAMP-dependent protein kinase A (PKA) and subsequent phosphorylation of AQP2 at Ser256. Consequently, the AQP2-containing vesicles fuse with the apical membrane, causing a dramatic increase in the membrane water permeability (Knepper and Inoue, 1997; Nishimoto et al., 1999). Several lines of evidence suggest the same V2R–AC–cAMP–AQP2 mechanism in the inner ear. (1) The V2R is expressed in the rat inner ear (Kitano et al., 1997). (2) The AC is expressed in the rat inner ear (Mees, 1984; Drescher et al., 2000) and activated by vasopressin in the guinea pig inner ear, leading to elevation of intracellular cAMP (Zenner and Zenner, 1979). The effect of vasopressin on the rat inner ear resulting in hydrops of the endolymphatic space is most likely caused by decreased fluid reabsorption in the endolymphatic sac epithelium and this decrease is mediated by V2R coupling to the cAMP pathway (Kumagami et al., 1998). (3) AQP2 is expressed in the mammalian cochlea (Mhatre et al., 2002) and AQP2 mRNA is elevated by vasopressin in the rat inner ear (Sawada et al., 2002). (4) Vesicle-mediated transport proteins such as vesicle-associated membrane protein 2, syntaxin-4, the small GTPase Rab3a and calcium/polycation sensing receptor exist in the rat inner ear (Beitz et al., 1999). Some data showed the presence of vasopressin V1a receptor mRNA in the stria vascularis (Minteguiaga et al., 1998) and endolymphatic sac (Furuta et al., 1999) of the rat inner ear. Further studies are necessary to clarify the function of vasopressin V1a receptor in the inner ear.

Other aquaporins were expressed in the inner ear including AQP1, AQP3, AQP4, AQP5, AQP6, AQP7 and AQP9. The application of vasopressin to Xenopus oocytes injected with AQP1 cRNA and 8-bromo-cAMP incubation with or direct injection into oocytes expressing AQP1 cRNA both increased the membrane permeability to water, suggesting that stimulation of AQP1 activity by vasopressin may involve a cAMP-dependent mechanism (Patil et al., 1997). AQP3 mRNA in the ipsilateral cochlea was up-regulated after unilateral intra-endolymphatic sac application of dexamethasone in the rat (Kitahara et al., 2003). AQP3 mRNA in the rat endolymphatic sac was significantly up-regulated in both dose- and time-dependent manners after intraperitoneally injections of steroids (Fukushima et al., 2004). To our knowledge, there are no reports about the effects of vasopressin on other aquaporins except AQP2.

The ion channels are well known to play an important part in the metabolism of water and electrolytes. Vasopressin increased the IKs channel current in the neonatal rat marginal cells, and the fact that 8-bromo-cAMP or 1-Deamino-D-arginine vasopressin also showed similar effects, suggesting the involvement of V2R and the subsequent activation of the cAMP signal pathway (Lee et al., 2001).

So vasopressin probably affects fluid balance in the inner ear via different receptors, aquaporins and ion channels involving complex multiple sites and pathways. Therefore, we investigate the effects of vasopressin on various gene expressions simultaneously in rat inner ear using cDNA microarray to elucidate the possible mechanism of the induced hydrolabyrinth, furthermore, the changes of aquaporin expression level were measured by RT-PCR.

2. Materials and methods

2.1. Endolymphatic hydrops model

Sixteen healthy Wistar rats (Chinese Academy of Medical Sciences) with a positive Preyer's reflex weighing 200–250 g were used in this experiment. Eight rats were intraperitoneally injected with 50 μg/kg arginine vasopressin (V9879, Sigma-Aldrich Co., St. Louis, MO) dissolved in normal saline once a day for one week. Eight control rats were intraperitoneally injected with normal saline. The rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and subsequently perfused with 4% paraformaldehyde via the left ventricle. The temporal bones were removed rapidly and a small hole was created in the apex and round window of the cochlea. Then the cochleae were placed in the 4% paraformaldehyde for 48 h at 4 °C. The specimens were decalcified in 10% EDTA solution for 14–21 days, dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in paraffin using

routine procedures. The cochleae were horizontally sectioned with an ultramicrotome at 5 μm in thickness. Sections were stained with hematoxylin and eosin (HE) and observed under a light microscope. The presence and degree of endolymphatic hypdrops was assessed according to the ratio of the cross-sectional area of the scala media and the scala vestibuli from the mid-modiolar sections of the cochlea in the second and third turns using Image-Pro plus 6.0 software. The rats were allowed free access to food and water. The experiments were approved by the Animal Care and Use Committee of Peking Union Medical University.

2.2. cDNA microarray

Sixteen Wistar rats were used in this microarray experiment. The rats were anesthetized and subsequently decapitated. The temporal bones were immediately dissected out and rapidly frozen in liquid nitrogen. Sixteen cochleae from vasopressin group or control group were pooled and submitted for total RNA extraction using the TRIzol reagent (Gibco BRL, Grand Island, NY) following the manufacturers' instructions. The yield of total RNA was 12.0 μg per cochlea in vasopressin group and 19.2 μg per cochlea in control group. The quantity and purity of total RNA were measured by spectrophotometry: A260/A280 of total RNA was 2.0 in vasopressin group and 1.7 in control group, respectively. The high quality of total RNA was validated by agarose gels.

The cDNAs were synthesized and labeled by reverse transcription in the presence of total RNA 30 μg, ddH_2O 23 μl, 500 mg/L T7-Oligo dT 5 μl, 10 mmol/L dNTP mix (Promega Co., Madison, WI) 4 μl, 200 U/μl Superscript II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) 2 μl, 1 mmol/L Cy5-dCTP (Amersham Phamacia Biotech, Piscataway, NJ) 3 μl for vasopressin group and 1 mmol/L Cy3-dCTP (Amersham Phamacia Biotech, Piscataway, NJ) 3 μl for control group. The two labeled fluorescent cDNAs samples were mixed and then checked with the ScanArray 4000 scanner (General Scanning Inc., Watertown, MA).

The BiostarR-40s cDNA microarray (United Gene Co., Shanghai, China) consists of 4096 probes including 4041 rat cDNA sequences, 20 housekeeping genes (internal control), 16 plant genes (negative control) and 19 blank solution spots without DNA (blank control). The BiostarR-40s microarrays were pre-hybridized with solution containing 0.5 mg/ml denatured salmon sperm DNA at 42 °C for 6 h. The fluorescent cDNAs mixtures were denatured at 95 °C for 2 min, and the denatured sample mixtures were then hybridized with the pre-hybridized BiostarR-40s chips at 42 °C for 18 h. The hybridized chips were washed for 10 min with a buffer containing 2 × standard saline citrate (SSC) and 0.2% sodium dodecyl sulfate (SDS), for 10 min with a buffer containing 0.1 × SSC and 0.2% SDS, and for 10 min with a buffer containing 0.1 × SSC, respectively.

Following hybridization, the dried arrays were scanned with the ScanArray 4000 scanner at wavelength 532 nm for Cy3 and 635 nm for Cy5 to determine the intensity of each fluorescent spot according to procedures developed by the manufacturer. Grid spot measurement and background value subtraction were performed using the GenePix Pro 3.0 software (Axon Instruments, Inc., Foster City, CA). Digitized gene expression values of Cy3 and Cy5 were normalized with a global mean correction coefficient and exported to an Excel spreadsheet. Ratio of Cy5 and Cy3 was computed for each spot on microarray. In the present study, differentially expressed gene after vasopressin injection was identified if the value of the ratio was >2 or <0.5 in each duplicate.

2.3. RT-PCR analysis

The results of AQP from the microarray studies were validated by RT-PCR. Reverse transcription: 5 μg of total RNA harvested from vasopressin group or control group was reversely transcribed into cDNA using 200 U/μl Superscript II reverse transcriptase 1 μl, 500 mg/L Oligo dT 1 μl and 10 mmol/L dNTP 1 μl in a total volume of 50 μl. Polymerase chain reaction: 5 μl of the RT product was assayed for AQP2 (GenBank Accession Number:NM_012909) and AQP7 (GenBank Accession Number: NM_019157) transcripts using rat AQP2 specific primers (sense primer: 5′GCAGAAGTCGGAGCAGCAT3′, antisense primer: 5′CGTCGGTGGAGGCAAAGAT3′) and AQP7 specific primers (sense primer: 5′GCTTCGTGGATGAGGTATT3′, antisense primer: 5′ACTTATGGGTAGGGTAGGTTT3′). The primers used for assaying AQP2 and AQP7 expression yielded a fragment of 468 bp and 457 bp, respectively. PCR was carried out in PCR9700 (Applied Biosystems, Foster City, CA) in a total volume of 50 μl with 10 mmol/L dNTP 1 μl, 50 pmol sense primer, 50 pmol antisense primer, and 1 U *Taq* DNA polymerase 1 μl. β-Actin primer used in positive control instead of AQP specific primer yielded a fragment of 318 bp. Amplification was carried out with an initial preincubation step of 5 min at 94 °C followed by 35 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 59 °C for AQP2, at 58 °C for AQP7 and at 55 °C for β-actin, and extension for 45 s at 72 °C. All reactions were performed in triplicate. Multiple pools of RNA samples were assayed simultaneously on the same PCR plate.

RT-PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and confirmed by the molecular size of the product. In this study, we compared the relative quantification of the target AQP mRNA transcript to a reference β-actin mRNA transcript between vasopressin-injected rats and control rats. The mRNA transcript expression was measured by band densitometry using Band Leader Application Version 3.00 (Magnitec Ltd., Israel). The expression level of AQP mRNA was equivalent to the ratio of band intensity of AQP mRNA

and β-actin mRNA. Statistical analysis was performed with SPSS for Windows Version 12.0.1 (SPSS Inc., Chicago, IL)using the unpaired *t*-test.

3. Results

3.1. Results of endolymphatic hydrops model

Fig. 1 shows typical pictures of the cochlea section in the two groups. Reissner's membrane, while almost straight without extension in the control rat, was markedly extended in the experimental rat intraperitoneally injected with vasopressin. The ratio of the cross-sectional area of the scala media and the scala vestibuli in the vasopressin-injected group was 0.75 ± 0.11, and the ratio in the saline-injected group was 0.49 ± 0.05, $P = 0.01$ ($n = 8$). Significant differences were obtained between the vasopressin-injected group and the saline-injected group ($P < 0.05$).

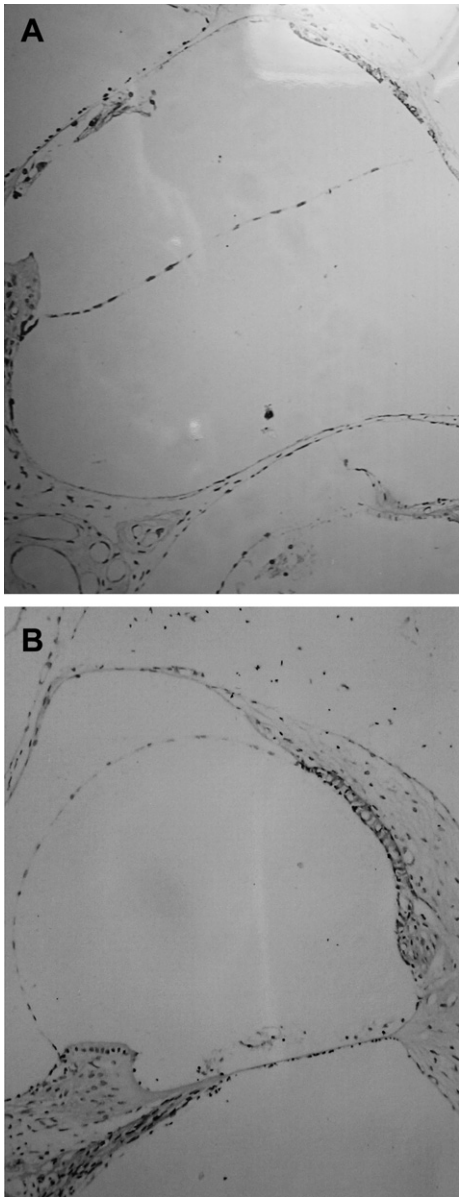

Fig. 1. Representative pictures of the cochlea in HE stain in the two groups: saline injected rat and vasopressin injected rat. Pictures show the second turn of the mid-modiolar section of the cochlea. A: straight Reissner's membrane in saline injected group; B: extended Reissner's membrane in vasopressin injected group. Distinct hydrops of the scala media was found in the vasopressin injected rat.

3.2. Results of cDNA microarray

Two hundred and twenty-six known differentially expressed genes were screened out in rat inner ear induced by vasopressin injection by cDNA microarray. Of the 226 genes, 18 transcripts were increased by 5-fold or more (see Table 1), and 7 transcripts were decreased to 0.2-fold or less (see Table 2). According to Rat Genome Database (http://rgd.mcw.edu/, This section last updated: April 17, 2006) and Gene Ontology (http://www.geneontology.org/, Last modified Saturday, 13-May-2006), 10 differentially expressed genes were identified that associate with cell signal transduction (see Table 3), 14 differentially expressed genes were identified that relate to ion transport (see Table 4), 7 differentially expressed genes were involved in vesicle-mediated transport (see Table 5), and 2 differentially expressed genes were AQP2 (Ratio = 3.5) and AQP7 (Ratio = 0.45).

3.3. Results of RT-PCR analysis of aquaporin

Fig. 2 shows the RT-PCR products obtained from the rat inner ear followed by 35 cycles were of the expected size of 468 bp. RT-PCR analyses revealed that AQP2 mRNA was expressed in the rat inner ear. AQP2 mRNA expression was increased in the vasopressin-injected rats. Fig. 3 shows the PCR products were of the expected size of 457 bp. RT-PCR results revealed that AQP7 mRNA was expressed in the rat inner ear and its expression was decreased in the vasopressin-injected rats.

To assess the effects of vasopressin on the expression level of AQP mRNA in the rat inner ear, a comparison of the ratio of band intensity of AQP mRNA and β-actin mRNA was made between the vasopressin-injected rats and saline-injected rats in Fig. 4. The ratios of AQP2 mRNA/β-actin mRNA and AQP7 mRNA/β-actin mRNA of the rat inner ear were 1.46 ± 0.31 and 0.21 ± 0.07 in the vasopressin-injected group ($n = 8$), and 0.97 ± 0.22 and 0.36 ± 0.11 in the saline-injected group ($n = 8$), respectively. The vasopressin injection resulted in a marked increase in the ratio of AQP2 mRNA and β-actin mRNA, and the increase was significant (*t*-test, $P < 0.05$). The vasopressin injection resulted in a marked decrease in the ratio of AQP7 mRNA and β-actin mRNA, and the decrease was significant (*t*-test, $P < 0.05$). From the above results of AQP2 and AQP7, there was agreement between cDNA array results and RT-PCR results.

Table 1
18 Differentially expressed genes were increased by 5-fold or more

Symbol	Name	Genbank_N	Ratio
Chn1	Chimerin (chimaerin) 1	NM_032083	22.697
Aldob	Aldolase B	M10149	22.111
Actc1	Actin alpha cardiac 1	X00306	20.490
Pak3	p21 (CDKN1A)-activated kinase 3	NM_019210	12.283
Acta2	Smooth muscle alpha-actin	M22757	11.624
Ddah1	Dimethylarginine dimethylaminohydrolase 1	NM_022297	8.338
Utrn	Utrophin	NM_013070	7.702
Ptprc	Protein tyrosine phosphatase, receptor type, C	Y00065	7.675
Scn3b	Sodium channel, voltage-gated, typeIII, beta	AF378093	7.027
Eno3	Enolase 3, beta	NM_012949	6.183
Dlad	DnaseII-like acid DNase	NM_021664	6.022
Tpm3	Tropomyosin 3, gamma	NM_057208	5.994
Ddit4l	DNA-damage-inducible transcript 4-like	NM_080399	5.936
Gnmt	Glycine N-methyltransferase	NM_017084	5.929
P2rx4	Purinergic receptor P2X, ligand-gated ion channel, 4	NM_031594	5.530
Ldhc	Lactate dehydrogenase 3, C chain	NM_017266	5.471
Cyp2b15	Cytochrome P450, family 2, subfamily b, polypeptide 15	NM_017156	5.392
Atp2a1	ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	NM_058213	5.386

Table 2
7 Differentially expressed genes were decreased to 0.2-fold or less

Symbol	Name	Genbank_N	Ratio
S100a9	S100 calcium binding protein A9 (calgranulin B)	NM_053587	0.124
Slc23a1	Solute carrier family 23 (nucleobase transporters), member 1	NM_017315	0.132
Fut2	Fucosyltransferase 2 (secretor status included)	NM_031635	0.153
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	NM_012942	0.156
Rps24	Ribosomal protein S24	NM_031112	0.169
Rpl41	Ribosomal protein L41	X82550	0.190
Eif5	Eukaryotic translation initiation factor 5	NM_020075	0.197

Table 3
Differentially expressed genes were identified that associate with cell signal transduction

Symbol	Name	Description	Genbank_N	Ratio
Gnas	GNAS complex locus	Alpha subunit of G protein Gs; couples G-protein coupled receptor binding to adenylate cyclase activation	NM_019132	4.125
Gnb1	Guanine nucleotide binding protein, beta 1	Component of heterotrimeric G-proteins; mediates activity of effector molecules and contributes to the specificity of G-protein receptor interaction	NM_030987	2.963
Plcd4	Phospholipase C, delta 4	Mediates signal transduction by generating two second messenger molecules, inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol, from phosphatidylinositol 4, 5-bisphosphate (PIP2)	U16655	2.798
Chn1	Chimerin (chimaerin) 1	May mediate neuronal signal transduction and function as GTPase-activating protein (GAP) for p21rac and phorbol ester receptor to increase the activity of a GTPase	NM_032083	22.697
Arf1	ADP-ribosylation factor 1	Catalyzes the reaction: $GTP + H_2O = GDP + phosphate$, decrease the activity of a small G protein	NM_022518	2.912
Arf6	ADP-ribosylation factor 6	Catalyzes the reaction: $GTP + H_2O = GDP + phosphate$	NM_024152	3.375
Rab7	RAB7, member RAS oncogene family	May act as a small GTPase	NM_023950	2.085
Pak3	p21 (CDKN1A)-activated kinase 3	Member of mitogen activated protein kinase kinase kinase (MAPKKK) displaying GTP-p21 mediated protein kinase activity	NM_019210	12.283
Ptprc	Protein tyrosine phosphatase, receptor type,C	Protein phosphatase displaying protein amino acid dephosphorylation	Y00065	7.675
Ppm1b	Protein phosphatase 1B, magnesium dependent, beta isoform	Protein phosphatase	NM_033096	3.027

Table 4
Differentially expressed genes were identified that relate to ion transport

Symbol	Name	Description	Genbank_ID	Ratio
Scn3b	Sodium channel, voltage-gated, type III, beta	Voltage-sensitive sodium channel auxiliary subunit	AF378093	7.027
Arf1	ADP-ribosylation factor 1	Plays a role in the activation of non-voltage-activated Na+-selective current	NM_022518	2.912
P2rx4	Purinergic receptor P2X, ligand-gated ion channel, 4	ATP-activated ionotropic receptor highly permeable to Ca2+	NM_031594	5.530
Gnb1	Guanine nucleotide binding protein, beta 1	May confer preferential receptor specificity to the voltage-sensitive calcium channel	NM_030987	2.963
Atp2a1	ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	Catalyzes ATP hydrolysis and calcium ion transport	NM_058213	5.386
Atp2a2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2	Sarcoplasmic reticulum Ca2+ ATPase involved in calcium transport from cytoplasm to endoplasmic reticulum	NM_017290	2.757
S100a9	S100 calcium binding protein A9 (calgranulin B)	Calcium binding protein associated with calcium movement	NM_053587	0.124
Pcyox1	Chloride ion pump-associated 55 kDa protein	Cl-pump subunit involved in outwardly directed Cl- transport	AF332142	0.209
Slc34a2	Solute carrier family 34 (sodium phosphate), member 2	May act as a sodium dependent phosphate transporter	NM_053380	3.537
Slc4a1	Solute carrier family 4, member 1	Transporter; functions as a chloride/carbonate exchanger	NM_012651	3.486
Slc2a4	Solute carrier family 2 (facilitated glucose transporter), member 4	Transports glucose	NM_012751	2.928
Slc7a7	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	Subunit of the y+LAT-1 amino acid transporter	NM_031341	2.039
Slc23a1	Solute carrier family 23 (nucleobase transporters), member 1	Mediates concentrative, high-affinity L-ascorbic acid transport which is driven by the Na+ electrochemical gradient	NM_017315	0.132
Slc12a1	Solute carrier family 12, member 1	Has bumetamide-sensitive Na+-K+-Cl- symporter activity	NM_019134	0.411

Table 5
Differentially expressed genes were involved in vesicle-mediated transport

Symbol	Name	Description	Genbank_ID	Ratio
Ap2b1	Adaptor-related protein complex 2, beta 1 subunit	Subunit of the clathrin-associated protein complex 2; component of the adaptor complexes which link clathrin to receptors in coated vesicles	M77246	3.262
Arf1	ADP-ribosylation factor 1	Regulates the formation of transport vesicles at many steps of the secretory and endocytic pathways	NM_022518	2.912
Arf6	ADP-ribosylation factor 6	Regulates the formation of transport vesicles	NM_024152	3.375
Dnch1	Dynein, cytoplasmic, heavy chain 1	Cytoplasmic motor protein that propels membranous organelles towards minus ends of polarized microtubules using ATP hydrolysis	D13896	2.675
Mlc3	Fast myosin alkali light chain	Contains some overlapping sequence with the myosin light chain 1 gene and provides differential regulation use of alternative promoters	NM_020104	2.382
Stx7	Syntaxin 7	Component of the soluble NSF attachment protein receptor complex; crucial for vesicle docking and fusion events with membrane; important for exocytosis and protein trafficking	NM_021869	0.480
Rab7	RAB7, member RAS oncogene family	Plays a role in the directed movement of proteins in a cell, including the movement of proteins between specific compartments or structures within a cell	NM_023950	2.085

4. Discussion

Two hundred and twenty-six known differentially expressed genes were screened out in rat inner ear induced by vasopressin injection by cDNA microarray. Of the 226 genes, 18 transcripts were increased by 5-fold or more, and 7 transcripts were decreased to 0.2-fold or less. Most of them were classified into signal transduction, ion movement and vesicle-mediated transport categories.

4.1. Effects of vasopressin on the expression of genes associated with cell signal transduction

Ten differentially expressed genes associated with cell signal transduction were increased and their detailed

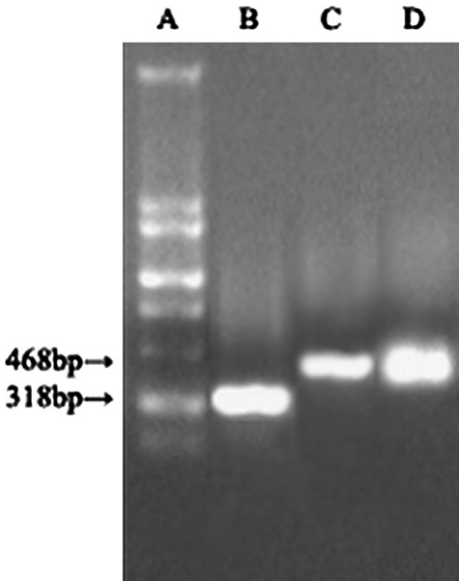

Fig. 2. RT-PCR determination of AQP2 mRNA in the inner ear of the vasopressin-injected and saline-injected rat. A: marker(DGL2000); B: β-actin; C: saline-injected; D: vasopressin-injected. RT-PCR results showed that AQP2 mRNA was expressed in the rat inner ear and its expression was stronger in the vasopressin-injected rat than in the saline-injected rat.

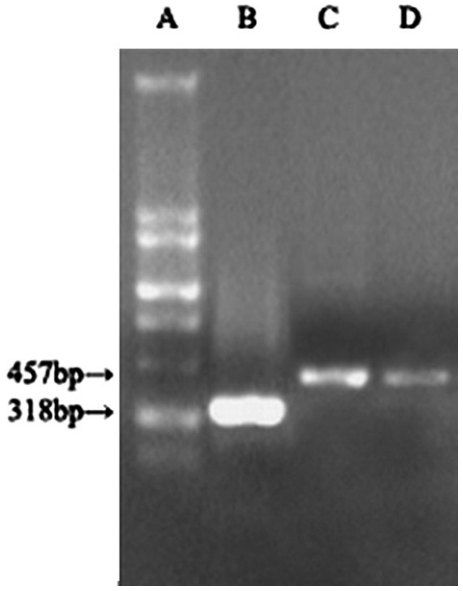

Fig. 3. RT-PCR determination of AQP7 mRNA in the inner ear of the vasopressin-injected and saline-injected rat. A: marker(DGL2000); B: β-actin; C: saline-injected; D: vasopressin-injected. RT-PCR results showed that AQP7 mRNA expression was weaker in the vasopressin-injected rat than in the saline-injected rat.

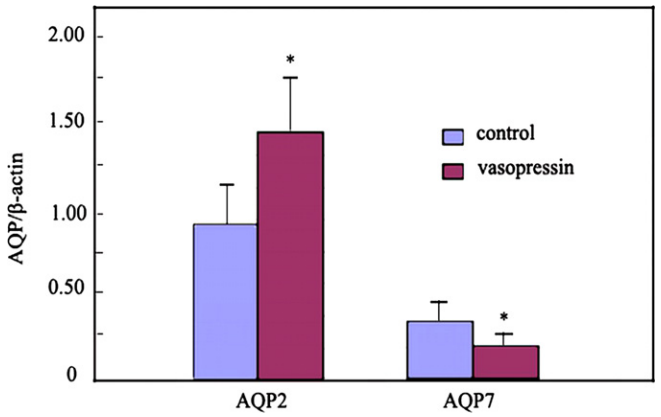

Fig. 4. A comparison of the ratio of band intensity of AQP mRNA and β-actin mRNA between the vasopressin-injected rats and saline-injected control rats. The ratios of AQP2 mRNA/β-actin mRNA and AQP7 mRNA/β-actin mRNA of the rat inner ear were 1.46 ± 0.31 and 0.21 ± 0.07 in the vasopressin-injected group ($n = 8$), and 0.97 ± 0.22 and 0.36 ± 0.11 in the saline-injected control group ($n = 8$), respectively. The ratio of AQP2 mRNA and β-actin mRNA was markedly increased by vasopressin treatment in the rat inner ear. The difference between the vasopressin-injected and saline-injected animals was significant (t-test, $P < 0.05$). The ratio of AQP7 mRNA and β-actin mRNA was markedly decreased by vasopressin treatment in the rat inner ear, and the difference was significant (t-test, $P < 0.05$).

functional roles were listed in Table 3. Gnas may couple G-protein coupled receptor binding to AC activation. This result is supported by the previous findings: (1) AC was present in the stria vascularis and semicircular canal epithelium of the inner ear (Ahlstrom et al., 1975; Bagger-Sjoback et al., 1980; Anniko et al., 1981; Mees, 1984; Schacht, 1985; Oudar et al., 1990; Drescher et al., 2000). (2) Vasopressin activated AC in the inner ear resulting in an increase in intracellular cAMP (Zenner and Zenner, 1979; Ferrary et al., 1991; Martin et al., 1994). In the kidney collecting duct, vasopressin bound to V2R, leading to activation of AC and elevation of cAMP, resulting in activation of PKA and subsequent phosphorylation of Ser256 of AQP2 in the cytosolic storge vesicles and their translocation to the apical membrane, causing an increase in the membrane water permeability (Knepper and Inoue, 1997; Nishimoto et al., 1999). Gnb1 contributes to the specificity of G-protein receptor interaction. Plcd4 mediates signal transduction by generating two second messenger molecules. So, vasopressin may affect signal transduction mediated by G-protein coupled receptor. Chn1 may function as GTPase-activating protein (GAP) to increase the activity of a GTPase. This result is consistent with that the rat renal inner medullary expression of neurofibromin, a member of the GAP family, was markedly increased at both mRNA and protein levels in response to long-term vasopressin infusion (Brooks et al., 2003). Arf1 and Arf6 decrease the activity of a small G protein. Rab7 may act as a small GTPase mediated signal transduction. The samll GTPase Rab3a was expressed in the endolymphatic sac of the rat inner ear (Beitz et al., 1999). Vasopressin may affect signal transduction also mediated by a small G protein. To our surprise, Pak3, Ptprc and Ppm1b mRNAs were increased. Pak3 is a family member of mitogen activated protein kinase kinase kinase (MAPKKK), Ptprc and Ppm1b are protein phosphatase. Further study is needed to investigate whether vasopressin directly activate MAPKKK.

4.2. Effects of vasopressin on the expression of genes related to ion transport

There is a close relationship between ion transport and fluid homeostasis in inner ear. Among the 14 differentially expressed genes related to ion transport, 10 genes were over expressed and 4 genes were under expressed. Their functional descriptions were listed in Table 4. Scn3b is a voltage-sensitive sodium channel auxiliary subunit. Arf1 plays a role in the activation of non-voltage-activated Na^+-selective current. P2rx4 is an ATP-activated ionotropic receptor and a channel highly permeable to Ca^{2+}. Previous study demonstrated that the P2X receptor was expressed in the sensorineural epithelium of rat cochlea (Cho et al., 2002). Gnb1 may confer preferential receptor specificity to the voltage-sensitive calcium channel. Atp2a1 directly drives the transport of calcium ion across a membrane. Atp2a2 is a sarcoplasmic reticulum Ca^{2+} ATPase involved in calcium transport from cytoplasm to endoplasmic reticulum. The Ca^{2+}-ATPase activity was found in the lateral cochlear wall of the guinea pig (Yoshihara and Igarashi, 1987), consistent with our result. S100a9 mRNA was decreased. S100a9 is a calcium binding protein associated with calcium movement. Pcyox1 mRNA was decreased. Pcyox1 is a Cl^- pump subunit involved in outwardly directed Cl^- transport. Slc12a1 mRNA was decreased. Slc12a1 has bumetamide-sensitive Na^+–K^+–Cl^- symporter activity. The rat renal medullary expression of the type 2 Na–K–2Cl cotransporter in mRNA and protein was decreased in response to vasopressin infusion (Brooks et al., 2003). Taken together, vasopressin may disturb fluid homeostasis in rat inner ear mediated by the change of gene expression relating to sodium channel, calcium channel, chloride channel and solute carrier family member.

4.3. Effects of vasopressin on the expression of genes involved in vesicle-mediated transport

The rat inner ear expressions of genes involved in vesicle-mediated transport were increased induced by vasopressin except Stx7 and their function were listed in Table 5. Ap2b1 is a component of the adaptor complexes which link clathrin to receptors in coated vesicles. Arf are thought to regulate the formation of transport vesicles. Dnch1 propels membranous organelles towards minus ends of polarized microtubules. Mlc3 provides differential regulation use of alternative promoters. These findings are in accordance with a previous study that numerous small vesicles were present in the apical cytoplasm of the dark cells of the frog semicircular canal epithelium induced by in vitro treatment with antidiuretic hormone (Oudar et al., 1991). The rat inner ear expression of Stx7 mRNA was decreased. Stx7 is crucial for vesicle docking and fusion events with membrane. It is interesting to find that the rat renal inner medullary expression of Stx2 was increased at mRNA level but was not increased at protein level in response to vasopressin infusion (Brooks et al., 2003). Rab7 may play a role in the directed movement of proteins between specific compartments or structures within a cell. As we mentioned above, the Rab3a was expressed in the endolymphatic sac of the rat inner ear (Beitz et al., 1999). Vasopressin mediated an inhibition of membrane turnover in the rat cultured endolymphatic sac epithelium. The decreased water permeability in the endolymphatic sac was modulated by inhibiting the translocation of AQP2 to the surface membrane (Kumagami et al., 1998). All these present and previous results lead to that vasopressin may affect water permeability in rat inner ear mediated by vesicle transport.

4.4. Effects of vasopressin on the expression of aquaporins

The rat inner ear expression of AQP2 was increased, consistent with the previous finding (Sawada et al., 2002). As we mentioned above, there exists V2R–AC–cAMP–AQP2 mechanism in renal collecting duct. The same mechanism may exist in the inner ear. It is likely that up-regulated expression of AQP2 mRNA in the stria vascularis caused by vasopressin increases water permeability to endolymph, resulting in an increased production of endolymph. The expression of AQP2 mRNA in the rat endolymphatic sac was increased and the pinocytosis from the rat endolymphatic sac was inhibited by vasopressin (Kumagami et al., 1998), resulting in a decrease of the endolymph absorption.

AQP7 was decreased in response to vasopressin, to our knowledge, this is the first report. It is currently believed that K^+ in the endolymph is recycled by way of supporting cells of the organ of Corti (Wangemann et al., 1995; Spicer and Schulte, 1996; Hibino et al., 1997). Along the pathway of the recycle, a variety of aquaporins are expressed in multiple types of cells. This pathway may provide a needed water permeability regulated by aquaporins to compensate for osmotic changes as a result of K^+ flow. It is likely that down-regulated expression of AQP7 mRNA in the cochlea caused by vasopressin induces decreased water permeability in the K^+ recycle pathway of the organ of Corti, resulting in a block of the endolymph circulation. In the endolymphatic sac, endolymph fluid is disposed of by the active pinocytosis against an osmotic gradient. And in the epithelium of the endolymphatic sac, the internalized hyperosmolar fluid is removed by ion secretion over the basolateral membrane and a concurrent water flux. It is likely that down-regulated expression of AQP7 mRNA in the endolymphatic sac caused by vasopressin induces decreased water permeability in the basolateral membrane, resulting in a decreased absorption of endolymph.

In conclusion, our results suggest that there are obvious differences in gene expression profiles in inner ear between vasopressin injected rats and normal control rats. It is a complicated pathophysiologic process that vasopressin disturbs fluid homeostasis in inner ear through signal transduction, ion transport, vesicle-mediated transport and aquaporins pathways resulting in hydrolabyrinth.

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References

Ahlstrom, P., Thalmann, I., Thalmann, R., Ise, I., 1975. Cyclic AMP and adenylate cyclase in the inner ear. Laryngoscope 85 (7), 1241–1258.

Anniko, M., Spangberg, M.L., Schacht, J., 1981. Adenylate cyclase activity in the fetal and the early postnatal inner ear of the mouse. Hear. Res. 4, 11–22.

Bagger-Sjoback, D., Filipek, C.S., Schacht, J., 1980. Characteristics and drug responses of cochlear and vestibular adenylate cyclase. Arch. Otorhinolaryngol. 228, 217–222.

Beitz, E., Kumagami, H., Krippeit-Drews, P., Ruppersberg, J.P., Schultz, J.E., 1999. Expression pattern of aquaporin water channels in the inner ear of the rat. The molecular basis for a water regulation system in the endolymphatic sac. Hear. Res. 132 (1–2), 76–84.

Brooks, H.L., Ageloff, S., Kwon, T.H., Brandt, W., Terris, J.M., Seth, A., Michea, L., Nielsen, S., Fenton, R., Knepper, M.A., 2003. cDNA array identification of genes regulated in rat renal medulla in response to vasopressin infusion. Am. J. Physiol. Renal. Physiol. 284 (1), F218–F228.

Cho, Y., Gong, T.W., Stover, T., Lomax, M.I., Altschuler, R.A., 2002. Gene expression profiles of the rat cochlea, cochlear nucleus, and inferior colliculus. J. Assoc. Res. Otolaryngol. 3 (1), 54–67.

Comacchio, F., Boggian, O., Poletto, E., Beghi, A., Martini, A., Rampazzo, A., 1992. Meniere's disease in congenital nephrogenic diabetes insipidus: report of two twins. Am. J. Otol. 13 (5), 477–481.

Drescher, M.J., Khan, K.M., Hatfield, J.S., Shakir, A.H., Drescher, D.G., 2000. Immunohistochemical localization of adenylyl cyclase isoforms in the lateral wall of the rat cochlea. Brain Res. Mol. Brain. Res. 76 (2), 289–298.

Dugue, B., Leppanen, E.A., Teppo, A.M., Fyhrquist, F., Grasbeck, R., 1993. Effects of psychological stress on plasma interleukins-1 beta and 6, C-reactive protein, tumour necrosis factor alpha, anti-diuretic hormone and serum cortisol. Scand. J. Clin. Lab. Invest. 53 (6), 555–561.

Ferrary, E., Oudar, O., Bernard, C., Friedlander, G., Feldmann, G., Sterkers, O., 1991. Adenylate cyclase in the semicircular canal. Hormonal stimulation and ultrastructural localization. Acta. Otolaryngol. 111 (2), 281–285.

Fukushima, M., Kitahara, T., Fuse, Y., Uno, Y., Doi, K., Kubo, T., 2004. Changes in aquaporin expression in the inner ear of the rat after i.p. injection of steroids. Acta Otolaryngol. Suppl. 553, 13–18.

Furuta, H., Sato, C., Kawaguchi, Y., Miyashita, T., Mori, N., 1999. Expression of mRNAs encoding hormone receptors in the endolymphatic sac of the rat. Acta Otolaryngol. 119 (1), 53–57.

Hibino, H., Horio, Y., Inanobe, A., Doi, K., Ito, M., Yamada, M., Gotow, T., Uchiyama, Y., Kawamura, M., Kubo, T., Kurachi, Y., 1997. An ATP-dependent inwardly rectifying potassium channel, KAB-2 (Kir4. 1), in cochlear stria vascularis of inner ear: its specific subcellular localization and correlation with the formation of endocochlear potential. J. Neurosci. 17 (12), 4711–4721.

Horii, A., Koike, K., Uno, A., Uno, Y., Kubo, T., 2001. Vestibular modulation of plasma vasopressin levels in rats. Brain. Res. 914 (1–2), 179–184.

Kitahara, T., Fukushima, M., Uno, Y., Mishiro, Y., Kubo, T., 2003. Up-regulation of cochlear aquaporin-3 mRNA expression after intra-endolymphatic sac application of dexamethasone. Neurol. Res. 25 (8), 865–870.

Kitano, H., Takeda, T., Pulec, J.L., Saadat, D., Uchida, K., 1994. The relationship between vasopressin and endolymphatic hydrops in the guinea pig. Ear. Nose. Throat. J. 73 (12), 921–925.

Kitano, H., Takeda, T., Suzuki, M., Kitanishi, T., Yazawa, Y., Kitajima, K., Kimura, H., Tooyama, I., 1997. Vasopressin and oxytocin receptor mRNAs are expressed in the rat inner ear. Neuroreport 8 (9–10), 2289–2292.

Knepper, M.A., Inoue, T., 1997. Regulation of aquaporin-2 water channel trafficking by vasopressin. Curr. Opin. Cell. Biol. 9 (4), 560–564.

Kumagami, H., Loewenheim, H., Beitz, E., Wild, K., Schwartz, H., Yamashita, K., Schultz, J., Paysan, J., Zenner, H.P., Ruppersberg, J.P., 1998. The effect of anti-diuretic hormone on the endolymphatic sac of the inner ear. Pflugers. Arch. 436 (6), 970–975.

Lee, J.H., Kim, J., Kim, S.J., 2001. Effect of vasopressin on marginal cells of neonatal rat cochlea in vitro. Acta Otolaryngol. 121 (8), 902–907.

Lim, J.S., Lange, M.E., Megerian, C.A., 2003. Serum antidiuretic hormone levels in patients with unilateral Meniere's disease. Laryngoscope 113 (8), 1321–1326.

Martin, F., Marianowski, R., Tu, T.Y., Herman, P., Tran Ba Huy, P., 1994. Modulation of cyclic AMP production by strial marginal cells from gerbil in culture. Hear. Res. 81 (1–2), 33–41.

Mees, K., 1984. Cytochemical localization of adenylcyclase in the lateral wall of the inner ear. Arch. Otorhinolaryngol. 240 (1), 55–61.

Mhatre, A.N., Jero, J., Chiappini, I., Bolasco, G., Barbara, M., Lalwani, A.K., 2002. Aquaporin-2 expression in the mammalian cochlea and investigation of its role in Meniere's disease. Hear. Res. 170 (1–2), 59–69.

Minteguiaga, C., Clerget, M.S., Tran Ba Huy, P., Elalouf, J.M., 1998. beta2-adrenergic but not vasopressin V2 receptor mRNAs are expressed in the stria vascularis of the rat inner ear. Pflugers. Arch. 436 (6), 940–947.

Nishimoto, G., Zelenina, M., Li, D., Yasui, M., Aperia, A., Nielsen, S., Nairn, A.C., 1999. Arginine vasopressin stimulates hosphorylation of aquaporin-2 in rat renal tissue. Am. J. Physiol. 276 (2 Pt 2), F254–F259.

Oudar, O., Ferrary, E., Feldmann, G., 1990. Adenylate cyclase and carbonic anhydrase in the semicircular canal epithelium of the frog Rana esculenta. An ultrastructural cytochemical localization. Cell. Tissue. Res. 262, 579–585.

Oudar, O., Ferrary, E., Feldmann, G., 1991. Antidiuretic-hormone-induced morphological changes in the ampullary epithelium of the frog semicircular canal. Eur. Arch. Otorhinolaryngol. 248 (7), 386–389.

Patil, R.V., Han, Z., Wax, M.B., 1997. Regulation of water channel activity of aquaporin 1 by arginine vasopressin and atrial natriuretic peptide. Biochem. Biophys. Res. Commun. 238 (2), 392–396.

Sawada, S., Takeda, T., Kitano, H., Takeuchi, S., Kakigi, A., Azuma, H., 2002. Aquaporin-2 regulation by vasopressin in the rat inner ear. Neuroreport 13 (9), 1127–1129.

Sawada, S., Takeda, T., Saito, H., 1997. Antidiuretic hormone and psychosomatic aspects in Meniere's disease. Acta Otolaryngol. Suppl. 528, 109–112.

Schacht, J., 1985. Hormonal regulation of adenylate cyclase in the stria vascularis of the mouse. Hear. Res. 20, 9–13.

Spicer, S.S., Schulte, B.A., 1996. The fine structure of spiral ligament cells relates to ion return to the stria and varies with place-frequency. Hear. Res. 100 (1–2), 80–100.

Takeda, T., Kakigi, A., Saito, H., 1995. Antidiuretic hormone (ADH) and endolymphatic hydrops. Acta Otolaryngol. Suppl. 519, 219–222.

Takeda, T., Takeda, S., Kitano, H., Okada, T., Kakigi, A., 2000. Endolymphatic hydrops induced by chronic administration of vasopressin. Hear. Res. 140 (1–2), 1–6.

Wangemann, P., Liu, J., Marcus, D.C., 1995. Ion transport mechanisms responsible for K^+ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro. Hear. Res. 84 (1–2), 19–29.

Yoshihara, T., Igarashi, M., 1987. Cytochemical localization of Ca^{2+}-ATPase activity in the lateral cochlear wall of the guinea pig. Arch. Otorhinolaryngol. 243, 395–400.

Zenner, H.P., Zenner, B., 1979. Vasopressin and isoproterenol activate adenylate cyclase in the guinea pig inner ear. Arch. Otorhinolaryngol. 222 (4), 275–283.