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Ⅵ. 資　　　料

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Effects of Lithium on Endolymph Homeostasis and Experimentally Induced Endolymphatic Hydrops

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Key Words
Lithium · Aquaporin 2 · Endolymph homeostasis · Ménière's disease · Vasopressin

Abstract
There is evidence to suggest that water homeostasis in the inner ear is regulated via the vasopressin (VP)-aquaporin 2 (AQP2) system in the same fashion as in the kidney. The VP-AQP2 system in the kidney is well known to be inhibited by lithium, resulting in polyuria due to a decrease in reabsorption of water in the collecting duct of the kidney. Therefore, lithium is also likely to inhibit the VP-AQP2 system in the inner ear, and consequently exert some influence on inner ear fluid homeostasis. In this study, we investigated the effects of lithium on AQP2 expression in the rat inner ear, and on the cochlear fluid volume in hydropic ears of guinea pigs. A quantitative PCR study revealed that lithium reduced AQP2 mRNA expression in the cochlea and endolymphatic sac. Lithium application also decreased the immunoreactivity of AQP2 in the cochlea and endolymphatic sac. In a morphological study, lithium intake significantly reduced endolymphatic hydrops dose-dependently. These results indicate that lithium acts on the VP-AQP2 system in the inner ear, consequently producing a dehydratic effect on the endolymphatic compartment.

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Introduction

Lithium has been used worldwide as a therapeutic agent in manic-depressive disorders [1]. Lithium treatment is associated with various serious side effects [2]. In particular, nephrogenic diabetes insipidus is commonly associated with lithium treatment [3]. Although the mechanism underlying lithium-induced polyuria is not yet fully understood, lithium may inhibit the activity of adenylate cyclase and thus prevent the production of cAMP [3] and subsequently the activity of protein kinase A, which are involved in the regulation of aquaporin (AQP)-2 expression as well as vesicular trafficking of AQP2 [4]. Actually, chronic intake of lithium has been experimentally confirmed to cause down-regulation in AQP2 protein expression and a marked decrease in immunoreactivity in the rat kidney medulla and cortex [5–7].

Various kinds of water channels exist in the inner ear. Recently, proteins and/or mRNAs of AQP1 [8–10], AQP2 [11–15], AQP3 [9, 11, 14], AQP4 [9, 11, 16, 17], AQP5 [11, 18], AQP6 [19], AQP7 [9, 11] and AQP9 [9] have been reported to be expressed in the rat cochlea and/or the rat endolymphatic sac. As to AQP2, we reported that mRNA of the vasopressin type 2 receptor (V2-R), through which vasopressin (VP) mediates AQP2 trafficking from intracellular vesicles to the apical plasma membrane, is expressed in the inner ear [20]. Furthermore, the expression of AQP2 mRNA in the inner ear is up-regu-

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lated by VP [12], and the expression of V2-R mRNA in the cochlea is down-regulated reversely [21]. Therefore, the molecular-biological lines of evidence support the novel view that water homeostasis in the inner ear is regulated in part via the VP-AQP2 system in the same fashion as in the kidney.

From the point of view that inner ear fluid homeostasis may be regulated via the VP-AQP2 system similarly to that of the kidney, lithium might inhibit the VP-AQP2 system in the inner ear, resulting in reduced AQP2 protein and mRNA expressions in the inner ear. It is also likely that the down-regulation of AQP protein and mRNA expressions reduces experimentally induced endolymphatic hydrops, as observed in the application of a V2-antagonist (OPC-31260) [22]. In the present study, molecular-biological and histochemical studies were performed to investigate the influence of lithium on the expressions of AQP2 protein and mRNA in the inner ear. In addition, the effects of lithium on endolymphatic hydrops were examined.

Materials and Methods

Two experiments were performed in the present study. In experiment 1, histochemical and molecular-biological studies were performed to investigate the effects of lithium on AQP2 protein and mRNA levels in the inner ear. In experiment 2, a morphological study was performed to quantitatively assess lithium-induced volumetric changes of the endolymphatic space in experimental endolymphatic hydrops. These experiments were approved by the Kochi Medical School Animal Care and Use Committee, which conforms to The Animal Welfare Act and the guiding principles for animal care produced by the Ministry of Education, Culture, Sports and Technology, Japan.

Experiment 1: Immunohistochemical and Molecular-Biological Study

Animals used were 10 BN rats weighing 200–300 g. Animals were divided into two groups of 5 control animals and 5 lithium-treated animals. Animals were fed in metabolic cages with free access to water (controls) or physiological saline (lithium-treated animals) for 4 weeks. Lithium-treated animals received lithium chow, in which lithium chloride (LiCl) was added to a standard rodent diet (CE-2, Clea Japan Inc., Tokyo) to yield a lithium concentration of 60 mmol/kg dry food, and control animals received a standard rodent diet without LiCl. A chronic intake of lithium produces severe depletion of sodium, subsequently resulting in fatal outcome. Therefore, adequate salt intake is required to avoid negative sodium balance in lithium-treated animals. An intake of physiological saline is one of the methods suitable for this purpose.

The immunohistochemical study was designed to investigate the expression of AQP2 protein in the cochlea in control and lithium-treated animals. The molecular-biological study was designed to investigate the effects of LiCl on AQP-2 mRNA expression in both the cochlea and endolymphatic sac by a quantitative analysis of real-time PCR.

Immunohistochemistry

Fixation and Single Labeling. One lithium-treated animal and one control animal were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused with phosphate-buffered saline (PBS), followed by a fixative (pH 7.4) containing 4% paraformaldehyde in PBS. Temporal bones were dissected and immersed in the fixative overnight, and then decalcified in 0.12 *M* ethylenediaminetetraacetic acid in PBS (pH 7.4) for 7 days. The cochleae were soaked in 15% sucrose in PBS at 4°C overnight, and were then embedded in Tissue Tec OCT Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Cochlea sections cut with a cryostat (HM 505E, MICROM) of 10 μm thickness were collected on slides. The sections were immersed in 3% sodium deoxycholate for 4 h at room temperature to enhance the permeability of tissues to antibodies and were then preincubated for 1 h in a solution containing 10% normal goat serum. The specimens were then exposed overnight at 4°C to rabbit antibodies specific for AQP2 (Cat. 178612, Lot B40067, Calbiochem, Germany), diluted 1:200. The sections were incubated for 6 h at room temperature in Alexa 546 goat antirabbit IgG (A-11035, Molecular Probes, Inc., Oregon, USA), diluted 1:100. Sections were washed extensively with PBS containing 10% normal goat serum after each antibody incubation.

Confocal Laser Microscopy. The sections incubated with Alexa 546 and 488 fluorescent antibodies were observed by a confocal laser scanning microscope system (LSM 410, Zeiss, Jena, Germany). Fluorescence of Alexa 546 was observed using an LSM 410 microscope equipped with a helium-neon laser (543 nm), a dicroic reflector (FT543) and an emission filter (FT570). Fluorescence of Alexa 488 was observed using an argon laser (488 nm), a dichroic reflector (FT510) and an emission filter (BP515-525).

Real-Time PCR and Quantitative Analysis of Real-Time PCR Data. Real-time PCR with the Roche LightCycler system (Roche Diagnostics, Biochemicals, Mannheim, Germany) was performed in a reaction mixture of 20 μl. This mixture contained 11.6 μl of H_2O, 2.4 μl of $MgCl_2$, 0.5 μmol of each primer solution, 2 μl of cDNA solution and 2 μl of LightCycler DNA Master SYBR Green I solution (Roche Diagnostics). The final concentration of $MgCl_2$ in the reaction mixture was 4 m*M*. The primers for the detection of AQP2 cDNA were: 5′-TGAGTTCTTGGCCACGCTCCTTT-3′ (sense; corresponding to nt 168–190) and 5′-ATGGAGAGG-GCAGGGCTACC-3′ (antisense; corresponding to nt 595–614) (GenBank, accession No. D13906). The pairs for β-actin were: 5′-TGGTGGGTATGGGTCAGAAGGACTC-3′ (sense; nt 131–155) and 5′-GATGGCTGGGGTGTTGAAGGTCTCA-3′ (antisense; nt 395–372) (GenBank, accession No. NM 031144). Real-time PCR was performed in glass capillaries with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 12 s at 60°C, and 10 s at 72°C. At the end of each cycle, fluorescence emitted by SYBR Green I was detected and the amount of PCR product was measured by fluorescence intensity. After the PCR reaction, the PCR product was confirmed to be specific to the primer pairs by means of a melting curve analysis and by the size of the product.

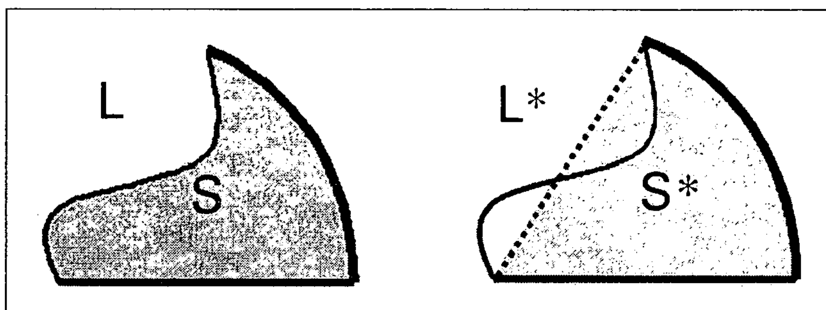

Fig. 1. Four parameters for the quantitative assessment of changes in endolymphatic space: (1) length of the extended Reissner's membrane (L, solid line); (2) original length of Reissner's membrane (L*, broken line); (3) cross-sectional area of the scala media (S, light gray area), enclosed by the distended Reissner's membrane, and (4) the cross-sectional area of the original scala media (S*, dark gray area), enclosed by a straight line segment (broken line). For equations see text.

Quantitative data analyses were conducted with Light Cycler analysis software (version 3.3, Roche Diagnostics). In this study, we compared the relative quantification of the target gene transcripts to a reference gene transcript (β-actin). For quantification, the crossing point was defined as the point at which the fluorescence rose appreciably above the background fluorescence. To quantify the target gene transcripts, standard curves for AQP2 and β-actin were obtained as follows. The control cDNA solution (100 μl) was prepared from 1 μg of rat kidney total RNA and diluted 1, 10, 100 and 1,000 times. The log concentration of the control cDNA and its crossing point were then plotted and the linearity of the plotted line was verified. The AQP2 mRNA level (M_{AQP2}) and β-actin mRNA level ($M\beta_{-actin}$) were determined as the cDNA concentration from each crossing point in the standard curves of AQP2 and β-actin.

The expression level of AQP2 mRNA was assessed by the ratio of AQP2 and β-actin mRNA ($M_{AQP2}/M\beta_{-actin}$ ratio). To evaluate the effect of Li administration on the expression of AQP2 mRNA in different tissues, $M_{AQP2}/M\beta_{-actin}$ values from tissue specimens (cochlea, endolymphatic sac, and kidney) of Li-treated animals were normalized to the mean $M_{AQP2}/M\beta_{-actin}$ values from the control animals. Statistical analysis was performed using the unpaired t test following the confirmation of equal variances by Levène's test. All data were expressed as means ± standard deviations.

Experiment 2: Morphological Study

Thirty Hartley guinea pigs with a positive Preyer's reflex and weighing about 300 g were used. All animals underwent electrocauterization of the endolymphatic sac in the left ear via an epidural occipital approach. Details of surgical procedures were described in a previous report [22].

Administration of Lithium, Perfusion and Fixation

One month after surgery, 30 Hartley guinea pigs (30 ears) were divided into three groups: a control group (10 animals), and 6- and 60-m*M* lithium groups (10 animals each). Animals were fed in metabolic cages with free access to water (controls) or physiological saline (lithium-treated groups) for 4 weeks. The control group was fed a standard diet, and the 6- and 60-m*M* lithium groups were fed lithium chow, in which LiCl was added to a standard diet to yield a lithium concentration of 6 and 60 mmol/kg dry foods, respectively.

After the 4-week treatment, all animals were perfused with physiological saline solution and fixed with 10% formalin under deep anesthesia with an intraperitoneal injection of pentobarbital sodium. We obtained the left temporal bones immediately following fixation and kept them in 10% formalin solution for 10 days or longer. They were decalcified with 5% trichloroacetic acid, dehydrated in increasing concentrations of alcohol, and then embedded in paraffin and celloidin. The prepared blocks were cut serially at 6 μm in the horizontal plane. The sections were stained with hematoxylin and eosin and studied under a light microscope.

Measurement Procedure

For the quantitative assessment of changes in endolymphatic space, the following four parameters (fig. 1) were measured in the basal, second, third and apical turns in the same way as previously described [22]: (1) the length of the extended Reissner's membrane (L), (2) the original length of Reissner's membrane (L*), (3) the cross-sectional area of the scala media (S), enclosed by the distended Reissner's membrane, and (4) the cross-sectional area of the original scala media (S*), enclosed by a straight line segment. The measuring system included a video camera, a computer, and a digitizer (Video Micro Meter VM-30, Olympus Co., Tokyo). The anatomical measurements were carried out in a blind fashion.

From these parameters, the ratios of the increase (%) in the length of Reissner's membrane (IR-L) and the cross-sectional area of the scala media (IR-S) of a total of four turns were calculated according to the following equations:

Ratio of the increase (%) in the length of Reissner's membrane (IR-L) =

$100 \times \Sigma(Lx - L^*x)/\Sigma L^*x$ (x = base, second, third, apex)

Ratio of the increase (%) in the cross-sectional area of the scala media (IR-S) =

$100 \times \Sigma(Sx - S^*x)/\Sigma S^*x$ (x = base, second, third, apex)

Results

Immunohistochemical Study

AQP2 protein is markedly expressed in the basolateral site of the stria vascularis and in the epithelial layer of the endolymphatic sac of a nontreated rat, but not in Reissner's membrane, hair cells, nor in the spiral ganglion

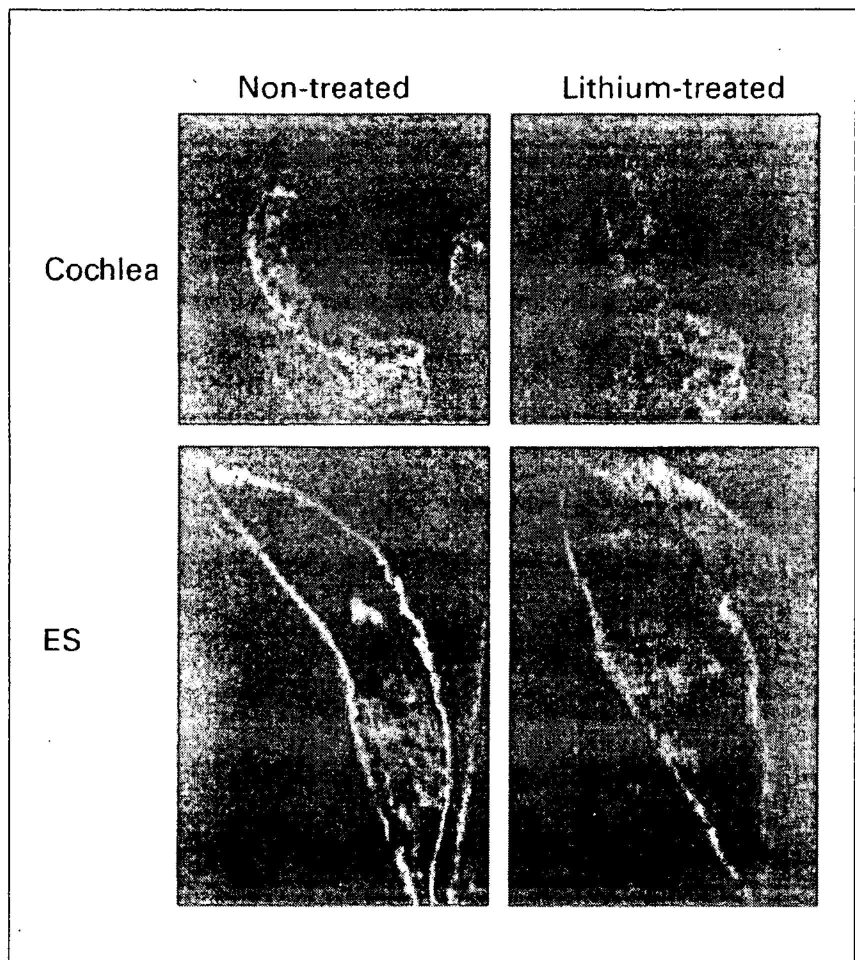

Fig. 2. Immunoreactivity of AQP2 protein in the cochlea and endolymphatic sac (ES). AQP2 protein was clearly expressed in the stria vascularis of the cochlea and in the epithelial layer of the ES. Lithium application decreased the immunoreactivity of AQP2 in both areas.

cells (fig. 2). The immunoreactivity of AQP2 both in the stria vascularis and the endolymphatic sac clearly diminished in a lithium-treated rat.

Real-Time PCR and Quantitative Analysis of Real-Time PCR Data

To assess the effects of lithium on the expression of AQP2 mRNA in the cochlea and endolymphatic sac, a comparison of the normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratio was made between the control rats and lithium-treated rats. The normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratios (means ± standard deviations) of the cochlea, the endolymphatic sac and the kidney were 1.00 ± 0.24, 1.00 ± 0.25 and 1.00 ± 0.09 in the control group (n = 4), and 0.06 ± 0.06, 0.42 ± 0.25 and 0.19 ± 0.14 in the lithium-treated group (n = 4), respectively (fig. 3). The lithium intake resulted in a marked decrease in the normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratio. The decreases in the control, 6- and 60-m*M* lithium groups were significant at $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively (t test).

Morphological Study

One animal of the 6-m*M* lithium group died during the period of feeding with lithium chow. The remaining 29 guinea pigs served as experimental animals. Figure 4 shows typical pictures of the cochlea in the three groups. Reissner's membrane is, regardless of the presence or absence of lithium treatment, remarkably extended due to the electro-cauterization of the endolymphatic sac. The most characteristic findings after lithium intake were the volumetric changes in the scala media. In the 6-m*M* lithium group, a slight folding of the distended Reissner's membrane was observed, but the decrease in the extent of endolymphatic hydrops was not so evident (fig. 4b).

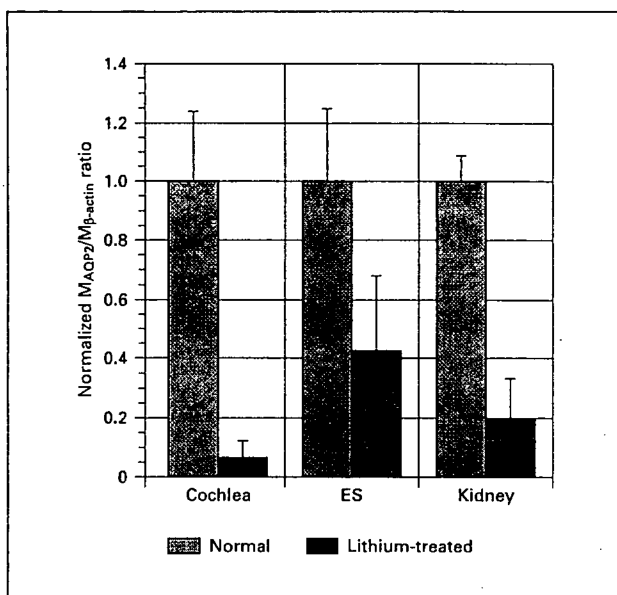

Fig. 3. Comparison of normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratios between normal and lithium-treated rats. The normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratios (means ± standard deviations) of the cochlea, endolymphatic sac (ES) and the kidney were 1.00 ± 0.24, 1.00 ± 0.25 and 1.00 ± 0.09 in the control group (n = 4), and 0.06 ± 0.06, 0.42 ± 0.25 and 0.19 ± 0.14 in the lithium-treated group (n = 4), respectively. Normalized $M_{AQP2}/M\beta_{\text{-actin}}$ ratios significantly decreased by lithium treatment in the cochlea, the endolymphatic sac and the kidney (t test, $p < 0.01$, 0.05, 0.001, respectively).

Collapse of the cochlear duct was noted in the 60-m*M* lithium group (fig. 4c). In the second turn especially, the extended Reissner's membrane was attached to the lateral wall of the cochlea.

Quantitative assessment was undertaken using the bivariate scattergrams of IR-L and IR-S of the three animal groups and the regression lines (fig. 5). Regression lines for both lithium-treated groups deviated downward from those for the control group, dose-dependently. Statistical analysis revealed that the deviation between the control and 6-m*M* lithium groups was statistically significant in constant terms of the regression line, but not in regression coefficients (ANCOVA, $p < 0.05$). The regression line for the 60 m*M* lithium-treated group significantly deviated from that for the control group (ANCOVA, $p < 0.001$). Significant differences were present both in constant terms of the regression lines and in the regression coefficients. A significant difference was also present between the 6- and 60-m*M* lithium groups (ANCOVA, $p < 0.001$). The downshift of the regression line indicates that the volume of the cochlear duct decreased compared with the length of Reissner's membrane. Therefore, the present result indicates that the volume of the scala media significantly decreased in the 6- and 60-m*M* lithium groups.

Discussion

In 1974, Thomsen et al. [23] first reported good results in an open pilot study of lithium treatment in Ménière's disease. In the same year, Lutz [24] also independently reported 3 cases of Ménière's disease with good responses to lithium carbonate. These lithium treatments in Ménière's disease were tried on the assumption that the hydrops of the endolymphatic space present in Ménière's disease was due to the defective transport of water and ions between the end- and perilymphatic spaces and that lithium, which has many biological effects on the transport of water and ions across membranes, corrects such defective water and ion transport in the inner ear. However, a double-blind crossover trial conducted by Thomsen et al. [25], pioneers in the field of lithium treatment of Ménière's disease, revealed that lithium treatment exerted only a placebo effect. Thereafter, lithium treatment in Ménière's disease has been completely discarded due to the results of this trial study.

In the present study, lithium intake caused a reduction in the immunoreactivity of AQP2 protein in the stria vascularis and endolymphatic sac. Furthermore, AQP2 mRNA expression of the cochlea and the endolymphatic sac also significantly decreased in lithium-treated rats. These results indicate that lithium inhibits the VP-AQP2 system in the inner ear as well as in the kidney. Since OPC-31260, an inhibitor of the VP-AQP system, acted as a dehydrating agent of the endolymphatic space in our previous study [22], it is likely that lithium may cause a reduction in endolymphatic volume. Indeed, the present morphological study indicated that lithium intake produced a dose-dependent reduction in the endolymphatic volume of the hydropic ear.

The study by Thomsen et al. [25] on lithium treatment of Ménière's disease was a nonrandomized trial, in which only 20 patients with Ménière's disease participated. The effect of lithium treatment was assessed by subjective symptoms including vestibular and cochlear symptoms. There was no description of audiometric findings during the treatment, which would indicate whether or not lithium treatment exerted any influence on endolymphatic hydrops. The previous favorable clinical response in an

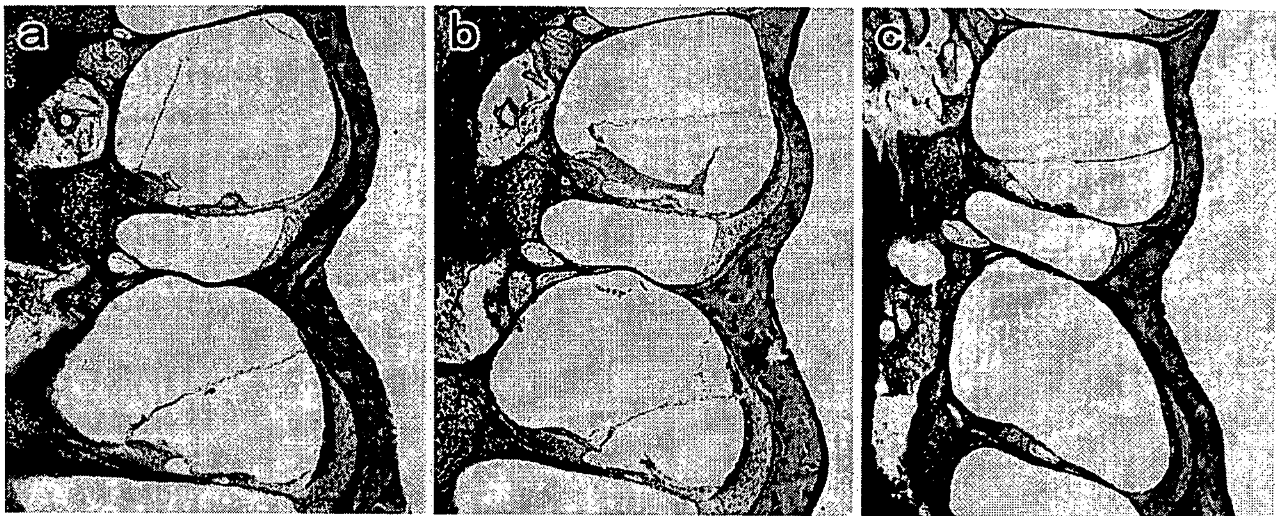

Fig. 4. Representative pictures of the cochlea in the three animal groups: a control animal (**a**), a 6-m*M* lithium animal (**b**), and a 60-m*M* lithium animal (**c**). The most characteristic findings after lithium intake were the volumetric changes in the scala media. In the 6 m*M*-lithium animal, a slight folding of the distended Reissner's membrane was observed, but the decrease in the extent of endolymphatic hydrops was not very evident. Reissner's membrane was markedly collapsed in the 60-m*M* lithium animal. The collapse was prominent in the second turn in this case.

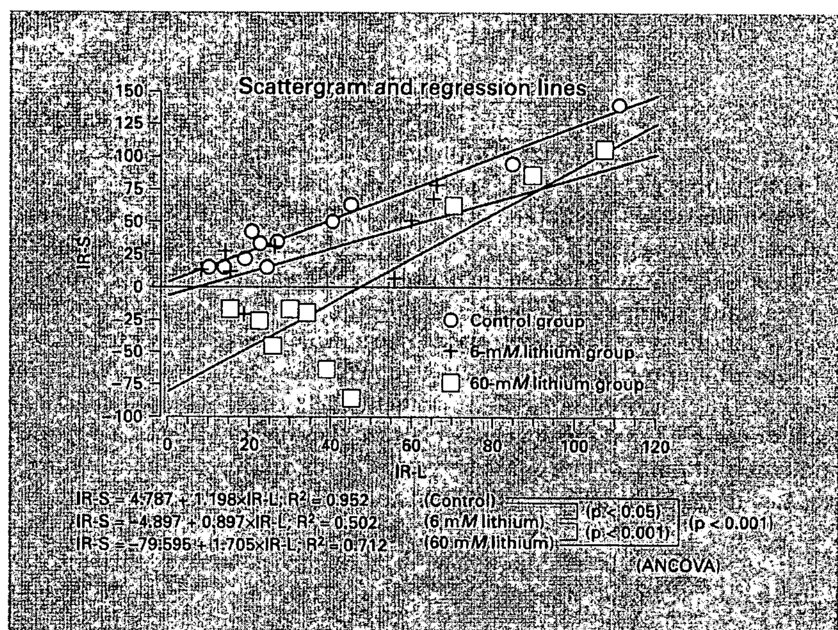

Fig. 5. Bivariate scattergrams of IR-L and IR-S of the three animal groups and the regression lines. Regression lines for the 6- and 60-m*M* lithium groups deviated downward from those for the control group in a dose-dependent fashion. Statistical analysis revealed that this deviation was significant. The downshift of the regression line indicates that the volume of the cochlear duct decreased compared with the length of Reissner's membrane. Therefore, the present result indicated that the volume of the scala media significantly decreased in the 6- and 60-m*M* lithium groups.

open trial of lithium treatment [23] may reflect the decompression of endolymphatic hydrops resulting from lithium-induced inhibition of the VP-AQP2 system in the inner ear. The present results call for the reassessment of lithium treatment.

Acknowledgments

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Research paper

A comparison of dehydration effects of V_2-antagonist (OPC-31260) on the inner ear between systemic and round window applications

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Abstract

V_2-antagonist (OPC-31260 (OPC)) application to the scala tympani reduced endolymphatic hydrops. In the present study, we investigated whether systemic administration or local infusion via the round window (RW application) of OPC would be more suitable for clinical use. In Experiment 1, the increase ratios of the cross-sectional area of the scala media of experimentally induced endolymphatic hydrops were quantitatively assessed among four groups of non-OPC application, RW application of xanthan gum, systemic application of OPC and RW application of OPC. In Experiment 2, the effects of systemic and RW applications of OPC on plasma vasopressin (p-VP) concentrations and plasma osmolality (p-OSM) were investigated. In Experiment 3, endocochlear DC potential (EP) was measured in guinea pigs with the RW application of OPC. Electron microscopic observations of the stria vascularis and the hair cells were also made. Both systemic and RW applications of OPC significantly reduced endolymphatic hydrops. However, systemic application resulted in the distension of the Reissner's membrane in the non-operated ear, which seemed to be caused by elevated p-VP levels resulting from the systemic application of OPC. In contrast, RW application of OPC produced no apparent toxic effects in the inner ear, as indicated electrophysiological or morphological changes. Thus, drug delivery via the round window is more useful for the clinical application of OPC for medical decompression.
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Keywords: Vasopressin; V_2-antagonist; OPC-31260; Endolymphatic hydrops; Meniere's disease

1. Introduction

Water is the major component of the body, and especially of the inner ear. The homeostasis of water in the inner ear is essential for maintaining the function of hearing and equilibrium. After aquaporin (AQP) water channels were discovered (Agre et al., 1993), it became clear that these channels likely play a crucial role in inner ear fluid homeostasis. Recently, proteins or mRNAs of AQP1 (Stankovic et al., 1995; Sawada et al., 2002), AQP2 (Kumagami et al., 1998; Beitz et al., 1999; Merves et al., 2000; Sawada et al., 2002; Fukushima et al., 2005), AQP3 (Beitz et al., 1999), AQP4 (Takumi et al., 1998; Minami et al., 1998), AQP5 (Beitz et al., 1999; Mhatre et al., 1999), AQP7 and AQP9 (Huang et al., 2002) were reported to be expressed in the inner ear. Regarding the vasopressin (VP)-mediated AQP2 system (VP-AQP2 system) in the inner ear, we reported that AQP2 mRNA expression was upregulated by the acute application of VP (Sawada et al., 2002), and downregulated by the local application of V_2-antagonist (OPC-31260) in the cochlea

Abbreviations: AQP, aquaporin; EH, endolymphatic hydrops; EP, endocochlear DC potential; IR-L, increase ratios of the length of Reissner's membrane; IR-S, cross-sectional area of the scala media; non-OPC application, animal group without any treatment except for the sac obliteration; p-OSM, plasma osmolarity; p-VP, plasma vasopressin; RW, round window; RW application, local infusion via the round window; S_{max}, maximally expanded cross-sectional area of the scala media, enclosed by the arc-shaped extended Reissner's membrane; SR-S, shrink ratio of the scala media; V_2-R, vasopressin type 2 receptor; OPC, OPC-31260, a selective V_2-R antagonist; VP, vasopressin; VP-AQP2 system, vasopressin-mediated AQP2 system

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as well as in the endolymphatic sac (Takeda et al., 2003), and that V_2-receptor (V_2-R) mRNA expression was downregulated by chronic application of VP (Kitano et al., 1999). The VP-AQP2 system in the kidney is well known to be regulated via cyclic AMP (Nielsen et al., 1999). We confirmed that the VP-AQP2 system in the inner ear was also regulated via cyclic AMP, as indicated by the fact that systemic application of lithium, an inhibitor of adenylate cyclase, reduced AQP2 protein expression both in the stria vascularis and in the endolymphatic sac (Fukushima et al., 2005). Since the stria vascularis and endolymphatic sac are thought to be the main sites of the secretion and/or absorption of endolymph (Sterkers et al., 1988), the homeostasis of endolymph is thought to be, in part at least, under the control of the AQP2 water regulation system mediated by vasopressin (VP-AQP2 system).

We reported that p-VP levels were elevated in cases of endolymphatic hydrops, including Meniere's disease (Takeda et al., 1995), and that chronic application of VP produced endolymphatic hydrops (EH) in guinea pigs (Takeda et al., 2000). Such clinical and experimental lines of evidence suggest that VP-induced over-accumulation of endolymph is one of the causative factors of the formation of EH, one of the characteristic morphological findings of Meniere's disease. EH formation, if due to the mal-regulation of the VP-AQP2 system in inner ear fluid, could be prevented by inhibitors of the VP-AQP2 system. Actually, the infusion of OPC-31260, a competitive antagonist of V_2-R (Yamamura et al., 1992), into the scala tympani reduced EH remarkably (Takeda et al., 2003). These experimental results indicate that the application of OPC will provide a new treatment strategy for Meniere's disease. However, direct infusion of OPC into the inner ear is not suitable for clinical application. In the present study, we investigated whether or not the same effect was also obtained by systemic administration or local infusion via the round window (RW application), which are better suited for clinical use. Furthermore, the presence of inner ear injuries resulting from the RW application of OPC was studied electrophysiologically and morphologically.

2. Materials and methods

Three experiments were performed. Experiment 1 was designed to morphologically investigate the effects of systemic and RW application of OPC on experimentally induced endolymphatic hydrops. Experiment 2 was designed to investigate the effects of systemic application of OPC on p-VP concentrations. In Experiment 3, the influence of RW application of OPC on endocochlear d-c potential (EP) was examined. Electron microscopic findings of the stria vascularis and the hair cells were also studied. These experiments were approved by the Kochi Medical School Animal Care and Use Committee, which conform to The Animal Welfare Act and the guiding principles for animal care formulated by the Ministry of Education, Culture, Sports and Technology, Japan.

2.1. Experiment 1

Thirty-five guinea pigs were used in this experiment. All animals received the surgical obliteration of the endolymphatic duct in the left ear and were maintained undisturbed and freely moving in individual cages with free access to water and standard chow in a quiet room. Four weeks after the obliteration, the animals were divided into four groups of non-OPC application ($n = 10$), RW application of xanthan gum ($n = 5$), systemic application of OPC ($n = 10$) and RW application of OPC ($n = 10$). In the non-OPC application group, animals were sacrificed without any treatment. In the systemic application group, OPC was given transorally four times at a dose of 100 mg/kg/8 h. Oral administration was performed via a rubber catheter introduced into the esophagus. Animals were sacrificed 6 h after the last administration. In the RW application groups, the round window was exposed via a retroauricular approach under general anesthesia with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). Xanthan gum dissolved in distilled water and a gelatinform of OPC (1 mg/animal) mixed with xanthan gum and distilled water were placed on the round window in RW application of xanthan gum and OPC, respectively, and then the retroauricular wound was closed with sutures. Thereafter, the animals were maintained for 30 h and then were sacrificed. All animals were subjected to quantitative assessment of volumetric changes of the endolymphatic space.

All animals were transcardially perfused with physiological saline solution under deep anesthesia by a peritoneal injection of pentobarbital, and fixation was performed with 10% formalin. The temporal bones of both sides were removed and postfixed in 10% formalin solution for 10 days or more. Thereafter, they were decalcified with 5% trichloroacetic acid and dehydrated in a graded ethanol series. They were embedded in paraffin and celloidin. The prepared blocks were cut horizontally into 6 μm sections. The sections were stained in hematoxylin and eosin, and observed under a light microscope.

2.1.1. Measurement procedure

For the quantitative assessment of changes of the endolymphatic space, the change ratios of the length of Reissner's membrane and the cross-sectional area of the scala media were measured from the mid-modiolar sections of the cochlea. Fundamentally, the measurement was performed as previously described (Takeda et al., 2000). In general, one specimen was used for the analysis of one cochlea. When the plane of the sections slightly deviated from the mid-modiolar axis, the sections that were cut closest to the mid-modiolar plane in individual turns were used. For this analysis, the following 4 parameters (Fig. 1) were measured in the basal, 2nd, 3rd and apical turns, not including the hook portion: (1) the length of

the extended Reissner's membrane (L: solid line), (2) the ideal length of Reissner's membrane (L^*: broken line). This line segment represents the normal position of the idealized Reissner's membrane, which connects the normal lateral attachment of Reissner's membrane at the upper margin of the stria vascularis to its normal medial attachment at the spiral limbus, (3) the cross-sectional area of the scala media (S: dark grey area), enclosed by the distended Reissner's membrane, and (4) the cross-sectional area of the original scala media (S^*: light gray area), enclosed by a straight line segment (broken line). The measuring system was composed of a video camera, a computer, and a digitizer (Video Micro Meter VM-30, Olympus Co., Tokyo). The anatomical measurements were carried out in a blind fashion. From these parameters, the increase ratios of the length of Reissner's membrane (IR-L) and the cross-sectional area of the scala media (IR-S) of a total of 4 turns were calculated according to the following equations:

$$\text{IR-L} = \sum_x L / \sum_x L_0 \quad (x\text{: base, second, third, apex})$$

$$\text{IR-S} = \sum_x S / \sum_x S_0 \quad (x\text{: base, second, third, apex})$$

In addition to these ratios, a shrink ratio of the scala media (SR-S) was introduced to quantitatively assess the dehydration effects of the OPC application among individual cochlea turns. The SR-S was defined as the ratio of the cross-sectional area of the scala media (S), enclosed by the folded Reissner's membrane to the maximum cross-sectional area of the scala media (S_{max}), as shown in Fig. 2. The S_{max} means the maximally expanded cross-sectional area of the scala media, enclosed by the arc-shaped extended Reissner's membrane, which is equal to the folded Reissner's membrane in length. In order to calculate SR-S in each of the basal, 2nd, 3rd apical turns, S and S_{max} of the medial and lateral cochlear ducts of the mid-modiolar sections were added in the individual turns, respectively,

$$\text{SR-S} = \sum_{m,l} S / \sum_{m,l} S_{max} \quad (m\text{: medial, } l\text{: lateral})$$

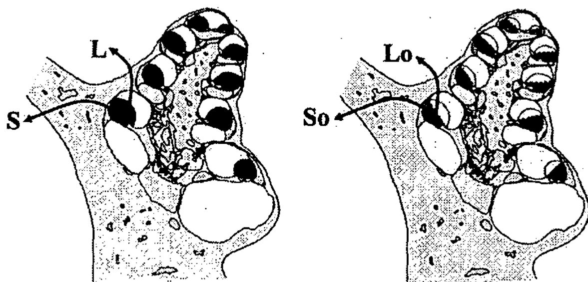

Fig. 1. Parameters for the quantitative assessment of scala media dilatation. These parameters were measured using a computerized digitizer (Video Micro Meter VM-30, Olympus Co., Tokyo).

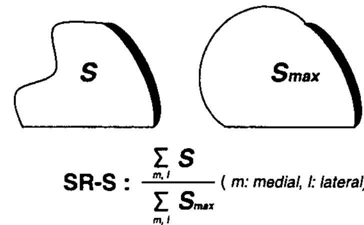

Fig. 2. Definition of the shrink ratio of the scala media (SR-S). The shrink ratios of individual cochlea turns were calculated using a formula employing the two parameters of S and S_{max}.

2.2. Experiment 2

Systemic administration of OPC is known to be associated with polyuria and dehydration, resulting in an elevation of p-VP (Fleeman et al., 2000). In this experiment, the effects of the methods of OPC administration on p-VP levels were investigated with the use of 15 guinea pigs. The animals were divided into three groups of non-OPC application, systemic application and RW application, including 5 animals each. Systemic and RW applications of OPC were performed in the same fashion as in Experiment 1. All animals were sacrificed for the measurement of p-VP concentrations and plasma osmolality (p-OSM). Animals with systemic application of OPC were sacrificed 6 h after the last administration, while animals with RW application were sacrificed 30 h after the application.

Blood samples were generally collected around 6 am, while animals were under light anesthesia established by an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). In this experiment, the animals were quickly decapitated at 0 min with the use of a guillotine, and trunk blood was collected in plastic tubes containing EDTA-Na. The blood volume collected was not less than 10 ml. Blood samples were immediately centrifuged at 4 °C, and plasma was stored at −20 °C until analyzed for VP. p-VP concentrations were measured by a radioimmunoassay of plasma extracted using C_{18} Sep-Pak cartridges (Water Associates, Milford, MA). The extracts were evaporated under a stream of air overnight. Analysis of arginine VP was performed with the use of the AVP-RIA kit 'Mitsubishi' (Mitsubishi Chemical Co., Tokyo). The detailed assay procedure was described previously (Takeda et al., 2000). p-OSM was measured in triplicate by assessing the freezing point depression using fresh heparinized samples (Advanced microosmometer 3MO, Advanced Instruments, Needham Heights, MA).

2.3. Experiment 3

Six guinea pigs were used in this experiment. All animals received an RW application of OPC on the left ear. Five of them were used for the measurement of EP. The remaining animal was used for a transmission electron microscopic (TEM) study.

2.3.1. EP measurement

An RW application of OPC was performed as described in Experiment 1. EP measurement was performed about 36 h after the application. Each animal was placed in the prone position under general anesthesia established with an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). First, the tympanic bulla of the left ear with an RW application of OPC was opened via a submandibular approach to expose the cochlea, and a hole of about 50-μm diameter was made in the bony wall over the scala media of the 2nd turn. A glass micropipette, which was beveled to a tip diameter of 10–15 μm and filled with artificial endolymph, was inserted into the scala media through the hole. The composition of the artificial endolymph was (in mM): KCl, 126; NaCl, 1; $KHCO_3$, 25; $MgCl_2$, 0.025; $CaCl_2$, 0.025; K_2HPO_4, 1.4; mannitol, 25 (pH 7.4), as described previously by Marcus et al. (1983). The micropipette was coupled to a microelectrode holder (MEH900S10: WPI Inc., Sarasota, FL, USA), connected to a differential electrometer (Electrometer FD-223; WPI Inc.). An Ag–AgCl wire placed in the neck muscle was also connected to the differential electrometer as reference. The differential electrometer was fed to a recorder system (PowerLab/4S, AD Instruments Pty Ltd., Castle Hill, Australia). Data were stored in a personal computer (Power Mac G5). In all experiments, EP was measured for around a 10-min period to confirm that EP was stable. Thereafter, EP on the opposite side was measured in the same fashion. After completing the EP measurement, all animals were sacrificed for a light-microscopic study to assess the dehydration effect of RW application of OPC on the endolymphatic space. The procedure for the assessment was the same as described in Experiment 1. The degree of hydrops was quantitatively assessed by determining the increase ratio of the scala media area in the mid-modiolar sections of the cochlea.

2.3.2. TEM study

Thirty hours after the RW application, the animal received an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). The animal was placed in a prone position with the head mounted in a head holder, and the RWs of both ears were exposed via a retroauricular approach. The cochleas of both ears were fixed by perilymphatic perfusion via the RW of fixative containing 1.0% paraformaldehyde, 1.5% glutaraldehyde and 1% OsO_4 in 0.07 M phosphate buffer (pH 7.2) with 3% sucrose for 15 min. Thereafter, the cochleas were quickly ablated, and the extirpated cochleas were immersed in the same fixative for an additional 60 min in the cold. After the fixation, the tissues were washed in 0.1 M phosphate buffer (pH 7.2) and dehydrated in a graded ethanol series and embedded in Spurr's resin.

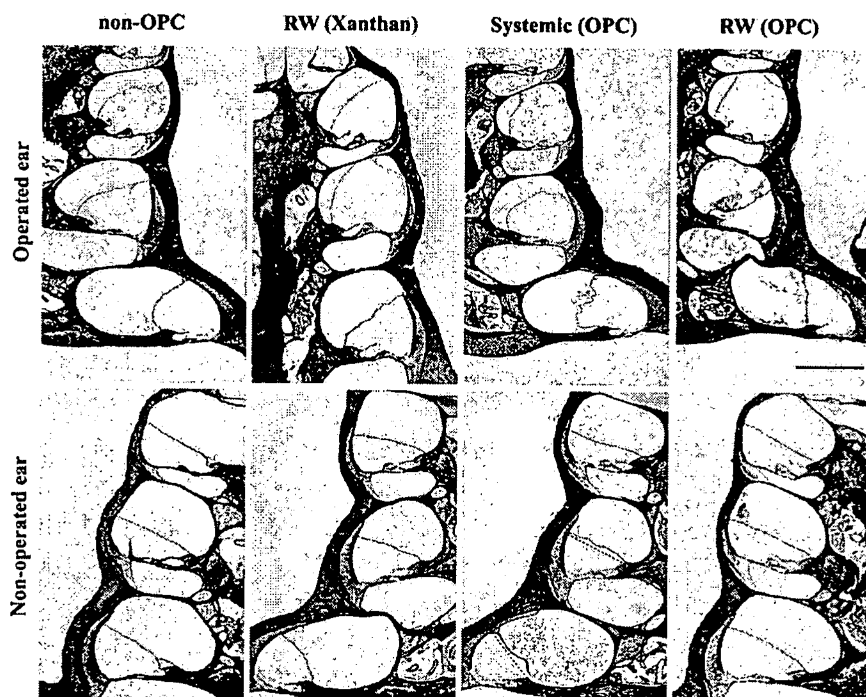

Fig. 3. Representative images of the cochlea in the four groups of no OPC-application, RW application of xanthan gum, systemic application of OPC and RW application of OPC (Experiment 1). In the groups of the non-OPC application and RW application of xanthan gum, the Reissner's membrane of the operated ear was remarkably extended and bulged into the scala vestibuli. However, the endolymphatic spaces were collapsed in both the systemic application and RW application. The horizontal bar indicates 500 μm. The following abbreviations are used in this figure. RW (xanthan): RW application of xanthan gum, systemic (OPC): systemic application of OPC. RW (OPC): RW application of OPC.

Ultrathin sections were stained with uranyl acetate and lead citrate, and observed using a Hitachi H700-H electron microscope at 75 kV.

3. Results

3.1. Morphological changes of endolymphatic space

Fig. 3 shows typical light microscopic images of four groups of non-OPC application, RW application of xanthan gum, systemic application of OPC and RW application of OPC. In the non-OPC application and RW application of xanthan gum, Reissner's membrane of the operated ear was remarkably extended and bulged into the scala vestibuli. The degree of expansion of the endolymphatic space seemed to be similar between the two groups. As shown in bivariate plots and regression lines of the IR-L and IR-S (Fig. 4a), statistical analysis indicated that RW application of xanthan gum did not have any influence on the endolymphatic space. In the systemic and RW applications of OPC, in contrast, distinct folding was observed in the extended Reissner's membrane. The endolymphatic spaces

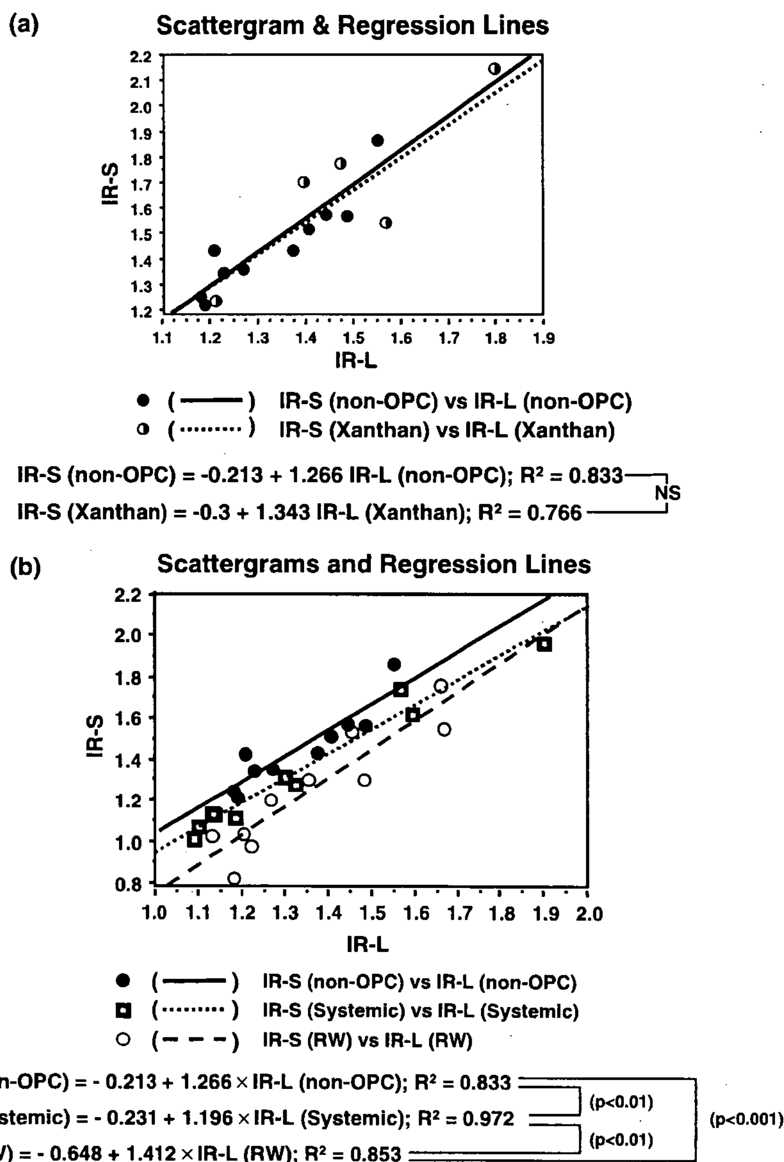

Fig. 4. Bivariate scattergram and regression lines of IR-S and IR-L (Experiment 1). (a) A comparison of regression lines between no OPC and RW application of OPC. The regression lines were not significantly different between non-OPC application and RW application of xanthan gum. This result indicated that RW application of xanthan gum did not have any influence on the endolymphatic space. (b) A comparison of the regression lines among three groups of non-OPC application, systemic application of OPC and RW application of OPC. The regression lines of the systemic and RW application groups significantly deviated from that of the non-OPC application group. The down-shift of the regression lines was also significant between the systemic and RW application groups. The down-shift means that the endolymphatic volume is small relative to the length of the extended Reissner's membrane.

were collapsed in both application groups. It should be noted that slight distension and folding of Reissner's membrane were also observed in the non-operated ear of animals with systemic application of OPC.

The bivariate scattergrams of the IR-L and IR-S of the hydropic ears of the three groups of non-OPC application, systemic application of OPC and RW application of OPC and the regression lines are presented in Fig. 4b. *R* squared (R^2) between IR-L and IR-S in the non-OPC, systemic and RW application groups was 0.833, 0.972, and 0.853, respectively. Statistically, there was a significant linear relationship between IR-L and IR-S in three groups (*t* test, $p < 0.0001$). Regression lines for RW and systemic applications deviated from the line for non-OPC application. Statistical analysis revealed that this deviation was significant (RW vs. non-OPC; ANCOVA, $p < 0.001$, systemic vs. non-OPC; ANCOVA, $p < 0.01$). Furthermore, the regression line for RW application significantly deviated downward compared to that for systemic application (ANCOVA, $p < 0.01$). A significant difference was present for the constant term of the regression line, but not for the regression coefficients. The down-shift of the regression line means that the volume of the cochlear duct was decreased compared with the extended length of the Reissner's membrane. SR-Ss of two groups of the systemic and RW applications of OPC were compared among individual turns of the basal, 2nd, 3rd and apical turns (Table 1). Both in the systemic application of OPC and in the RW application of OPC, differences in SR-Ss were not statistically significant among the basal, 2nd, 3rd and apical turns (one-way ANOVA).

As to the non-obliterated ears, slight bulging of the endolymphatic space was observed in the group with systemic application of OPC. Table 2 shows means and standard deviations (SDs) of IR-L and IR-S of the non-obliterated ears. The means of IR-Ss were not different among three application groups, but the means of IR-Ls were significantly different between the non-OPC and systemic application groups (one-way ANOVA, $p < 0.01$).

3.2. Experiment 2

Table 3 presents the means and SDs of p-VP concentrations and p-OSM. p-VP levels (mean ± SD) were 1.8 ± 0.4 pg/ml ($n = 5$) in non-OPC application animals, 19.9 ± 5.0 pg/ml ($n = 5$) in animals with systemic application of OPC, 1.4 ± 0.4 pg/ml in animals with RW application of OPC. The differences between non-OPC and systemic application, and between systemic and RW application, were significant ($p < 0.05$, Cochran–Cox test). There was no significant difference between the non-OPC and RW application groups (Cochran–Cox test). The p-OSM levels (mean ± SD) were 294.0 ± 4.0 mOsm/kg in non-OPC application animals, 360.8 ± 31.4 mOsm/kg in animals with systemic application of OPC, and 291.4 ± 3.1 mOsm/kg in animals with RW application of OPC, respectively. The differences between the non-OPC and systemic application groups and between the systemic and RW application groups were significant ($p < 0.05$, Cochran–Cox test). There was no significant difference between the non-OPC and RW application groups (Cochran–Cox test).

Table 1
A comparison of shrink ratios among individual cochlear turns in the two groups of the systemic and RW applications of OPC (Experiment 1)

	Basal Turn	2nd Turn	3rd Turn	Apical Turn
Systemic Appl. of OPC	0.74±0.08	0.78±0.04	0.79±0.03	0.79±0.05
	N.S. (among all turns)			
RW Appl. Of OPC	0.71±0.13	0.66±0.14	0.65±0.19	0.71±0.14
	N.S. (among all turns)			

SR-Ss did not show significant differences among individual cochlear turns in RW application of OPC or in systemic application of OPC (one-way ANOVA).

Table 2
Means and SDs of IR-L and IR-S of non-obliterated ears (Experiment 1)

	non-OPC	RW	Systemic
IR-L	1.02 ± 0.02	1.04±0.02	1.09 ± 0.04
	non-OPC vs. RW: NS; RW vs. Systemic: NS; non-OPC vs. Systemic: (p<0.01)		
IR-S	1.07 ± 0.03	1.07± 0.07	1.07± 0.16
	NS		

Means of IR-Ss were not significantly different among three application groups, but IR-Ls were significantly different between the non-OPC and systemic application groups (one-way ANOVA, $p < 0.01$).

Table 3
A comparison of p-VP and p-OSM among three groups of non-OPC application, systemic application of OPC and RW application of OPC (Experiment 2)

	non-OPC application	Systemic application	RW application
p-VP (pg/ml)	1.8±0.4 (n=5)	19.9± 5.0 (n=5)	1.4 ± 0.4 (n=5)
	non-OPC vs. Systemic: *; Systemic vs. RW: *; non-OPC vs. RW: NS		
p-OSM (mOsm/kg)	294.0±4.0 (n=5)	360.8 ± 31.4(n=5)	291.4±3.1 (n=5)
	non-OPC vs. Systemic: *; Systemic vs. RW: *; non-OPC vs. RW: NS		

Both p-VP and p-OSM showed a significant difference between the non-OPC application and systemic application of OPC and between the systemic application of OPC and RW application of OPC. (* $p < 0.05$, Cochran–Cox test).

3.3. Experiment 3

3.3.1. EP measurement

EP was stable at around 80 mV in both the ear with RW application of OPC and the opposite ear with no treatment. The EP value (mean ± SD) was 83.7 ± 3.04 mV in the ears with RW application of OPC, and 82.1 ± 2.25 mV in the untreated ears. The difference was not significant (paired Student's *t* test). Morphological studies revealed that collapse occurred in the endolymphatic space of the ear with RW application (Fig. 5). IR-S (mean ± SD) was 0.87 ±

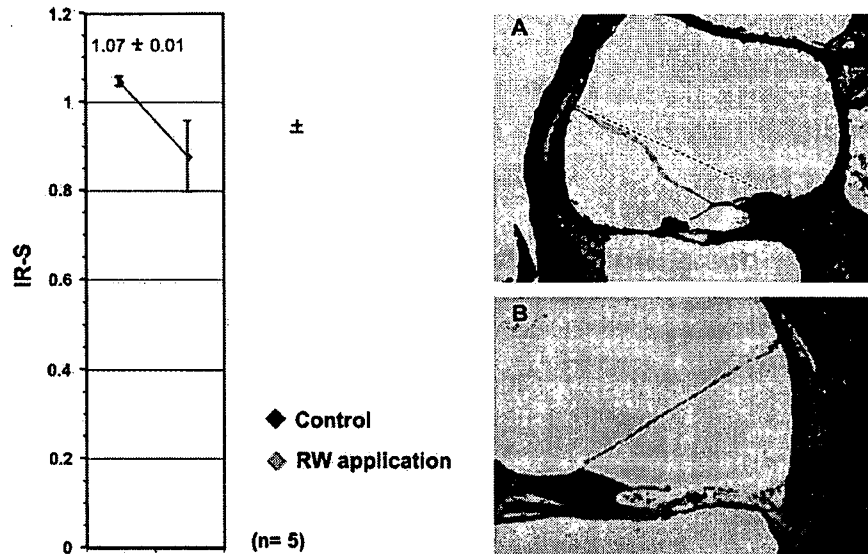

Fig. 5. Light microscopic images of the cochlea and volumetric changes of the endolymphatic spaces in ears with EP measurement (Experiment 3). Collapse was morphologically and quantitatively evident in the ear with RW application of OPC.

0.08 in the ears with RW application, and 1.04 ± 0.01 in the opposite ears with no treatment, respectively (paired Student's t test, $p < 0.01$).

3.3.2. TEM study

Fig. 6 shows transmission electron microscopic images of the stria vascularis (**6a**) and the hair cells (**6b**) in the ear with RW application of OPC. A fair number of autophagosomes were seen in the marginal cells. However, vacuolar degeneration was not detected. The intercellular spaces were tight, and lysosomes were rarely observed. No blebs were seen at the apical membrane. In the stria vascularis, there was no significant morphological difference between the ears with RW application of OPC and the opposite ears without any treatment. As for the hair cells, neither outer nor inner hair cells were missing, nor were they swollen or shrunken. Stereocilia were present and showed a normal appearance. No intracellular changes were observed.

4. Discussion

It is generally accepted that the formation of EH in Meniere's disease is retention hydrops, caused by malabsorption of the endolymphatic sac. This assumption is based on the morphological findings that the endolymphatic sac and duct are poorly developed (Shambaugh et al., 1969; Takeda et al., 1997), or otherwise fibrotic in

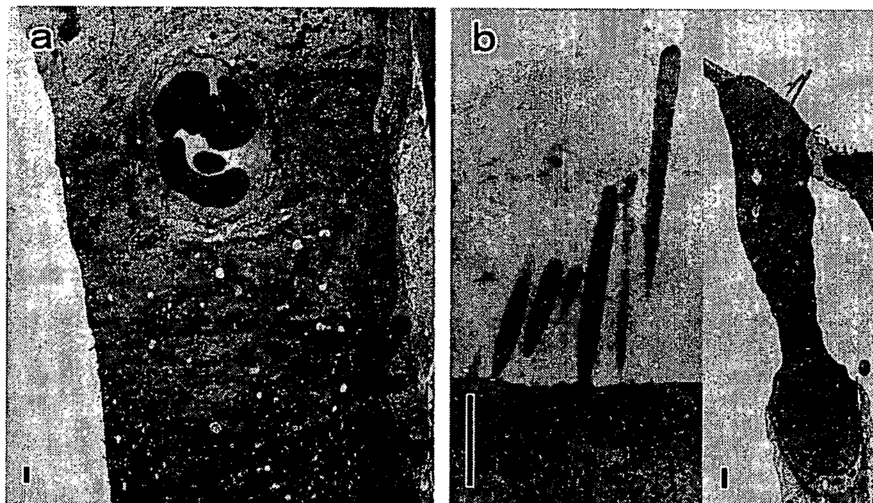

Fig. 6. Transmission electron microscopic images of the stria vascularis (Experiment 3). (a) The stria vascularis. Autophagosomes were seen in the marginal cells in the ear with RW-application of OPC. However, there was no significant morphological difference between the ears with RW application of OPC and the opposite ears with no treatment. (b) The outer hair cells. Stereocilia were present, and showed a normal appearance. No intracellular changes were observed. Vertical bars indicate 1 μm.