that radical scavengers may therefore be an effective form of treatment. We have already examined the possibility that both prevention and treatment of sensorineural hearing loss can be effected by controlling free radicals, and that radical scavengers may be effective for the treatment of hearing loss caused by MD [12] and cisplatin [13]. The efficacy of radical scavengers was also noted in the treatment of idiopathic sudden hearing loss [14]. Encouraged by these results, we undertook a pilot study to establish whether radical scavengers can be used to treat ARHL. Our results were both successful and encouraging [15]. However, that study included cases with asymmetrical hearing loss, especially in the low frequencies. In order to avoid any cause of hearing loss other than ARHL, we selected patients even more carefully and increased their number, which gave us more precise findings about the treatment of sensorineural hearing loss in elderly patients [16].

Subjects and methods

Subjects

The study subjects were recruited from a group of patients who complained of age-accompanying hearing impairment but who had no other cause of hearing loss, such as otitis media, MD, acoustic trauma, or acoustic tumour, from June 2005 to July 2007. They were scheduled for treatment of their hearing impairment at the Department of Otolaryngology in Hiroshima University Hospital or at North Fuchu City Hospital, Hiroshima. All the patients received a detailed explanation of the study, including risks and possible benefits, and their informed consent was obtained. The average age of the 46 patients (10 males and 36 females) was 76.7 years (range 70–91 years). They were given rebamipide (300 mg/day), vitamin C (600 mg/day), and α-lipoic acid (60 mg/day) for at least 8 weeks. The treatment was tapered off if and when requested by a patient. The effects on hearing and general health as well as possible side effects were investigated.

Evaluation of hearing

Determination of hearing change was based on pure-tone hearing levels at 125, 250, 500, 1000, 2000, 4000, and 8000 Hz and was accomplished by comparing the pretreatment hearing level with that after 8 weeks of treatment or with the final hearing level. An increase of $\geq$10 dB was deemed to denote a clinically significant improvement, a decrease or an increase $<$10 dB represented no change, and a decrease of $\geq$10 dB indicated deterioration.

Results

The average duration of treatment with radical scavengers during the study was 12.9 weeks (range 8–52 weeks). Two typical cases are illustrated in Figures 1 and 2.

The treatment outcome regarding hearing was evaluated in 92 ears. The average pure-tone hearing level threshold in patients during pretreatment was 48.7±11.23 (mean±SD) dB, which differed significantly from the average thresholds following radical scavenger therapy: 43.9±10.63 dB after 8weeks ($p<0.001$) and 43.5±10.67 dB at final observation ($p<0.001$). Hearing levels after 8 weeks of treatment as well as the final hearing levels had improved significantly compared with pretreatment at 125 Hz (44.3±13.63→6.9±14.48→36.7±14.09 dB; pretreatment → after 8 weeks→ final) ($p<0.001$), 250 Hz (42.8±15.03→35.8±13.79→35.4±13.74 dB) ($p<0.001$), 500 Hz (40.4±13.56→34.3±13.06→33.9±12.69 dB) ($p<0.001$), 1000 Hz (38.8±12.78→35.6±13.17→35.4±13.06 dB) ($p<0.001$), 2000 Hz (45.0±12.73→43.4±12.67→43.4±12.56 dB) ($p<0.05$), 4000 Hz (54.8±14.74→51.9±13.58→51.7±14.21 dB) ($p<0.001$), and 8000 Hz (74.9±17.08→69.1±15.89→68.3±15.90 dB) ($p<0.001$) (Figure 3).

At the final observation, 40 (43.5%) ears showed a clinically significant improvement of hearing ($\geq$10 dB), 49 were unchanged, and 3 were worse at 125 Hz; 38 (41.3%) ears were improved, 50 unchanged, and 4 worse at 250 Hz; 34 (37.0%) ears were improved, 56 unchanged, and 2 worse at 500 Hz; 22 (23.9%) were improved, 64 unchanged, and 6 worse at 1000 Hz; 9 (9.8%) ears were improved, 78 unchanged, and 5 worse at 2000 Hz; 21 (22.8%) ears were improved, 68 unchanged, and

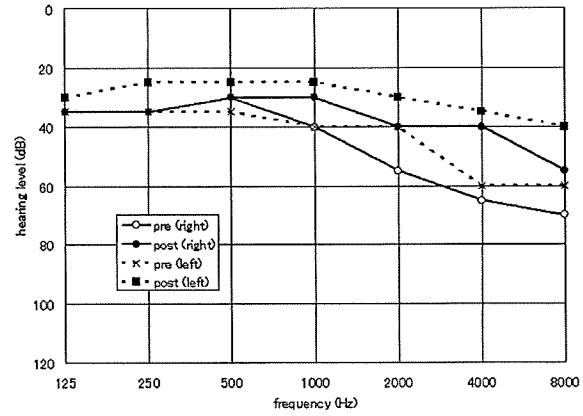

Figure 1. Case 1. A 73-year-old male complained of bilateral hearing loss. The audiogram showed steep high-frequency hearing loss. Amelioration of hearing was obtained after radical scavenger therapy. An ≈15 dB improvement was obtained at high frequencies.

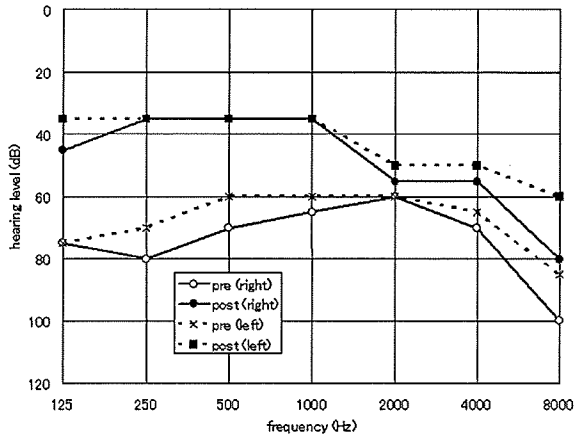

Figure 2. Case 2. An 84-year-old female complained of bilateral hearing loss. The audiogram showed a rather 'flat' type of hearing loss. Hearing improved gradually after radical scavengers were administered. An ≈20 dB improvement was obtained at all frequencies.

3 worse at 4000 Hz; 34 (37.0%) ears were improved, 55 unchanged, and 3 worse at 8000 Hz (Figure 4).

The correlation between the pretreatment hearing level and the change in hearing level was calculated for each ear at different frequencies. The improvement in hearing was greater at low frequencies (125, 250, and 500 Hz) and at 8000 Hz. However, there was little change in hearing before and after the radical scavenger therapy at 1000, 2000, and 4000 Hz. Moreover, significant correlations between pretreatment hearing levels and changes in hearing were noticed at 125 Hz ($p<0.001$), 250 Hz ($p<0.001$), 500 Hz ($p<0.001$), 1000 Hz ($p<0.01$), 2000 Hz ($p<0.05$), 4000 Hz ($p<0.01$), 8000 Hz ($p<0.001$), and average ($p<0.001$) (Figure 5).

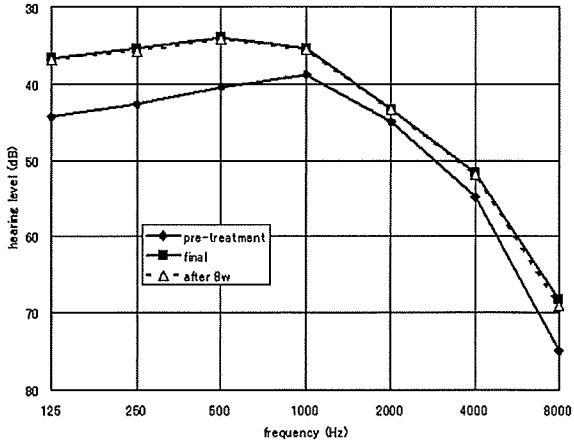

Figure 3. After 8 weeks of treatment, hearing levels improved significantly compared with pretreatment at all frequencies. The final hearing levels were also significantly improved.

Steep audiogram vs flat audiogram

To evaluate the treatment results for ARHL more strictly, analysis was also made between the patients with a steep type audiogram (steep group without hearing loss at low frequencies; excluding patients with ≥50dB hearing level at 125, 250, or 500 Hz) and the patients with a flat type audiogram (flat group with hearing loss at low frequencies; patients with ≥50 dB hearing level at 125, 250, or 500 Hz). The 'steep' group comprised 23 patients (6 males, 17 females) whose average age was 76 years (range 70–88 years). The average duration of treatment with radical scavengers during the study was 12.6 weeks (range 8–52 weeks). The treatment outcome regarding hearing was evaluated in 46 ears. The average pure-tone hearing level threshold in patients during pretreatment was 42.5±7.87 dB, i.e. differed significantly from the average thresholds following radical scavenger therapy: 38.2±8.06 dB at final observation ($p<0.001$). The final hearing levels had improved significantly compared with pretreatment at 125 Hz (35.4±7.73→29.3±10.41 dB; pretreatment→final) ($p<0.001$), 250 Hz (33.6±7.86→27.1±8.34 dB) ($p<0.01$), 500 Hz (32.6±8.48→27.1±8.60 dB) ($p<0.001$), 1000 Hz (33.4±10.90→30.2±11.59 dB) ($p<0.01$), 2000 Hz(42.0±12.63→40.2±13.25 dB) ($p<0.05$), and 8000 Hz (69.3±14.70→63.8±14.57 dB) ($p<0.001$), but were unchanged at 4000 Hz (51.3±13.88→49.2±13.94 dB) (Figure 6).

At the final observation, 17 (37.0%) ears showed a clinically significant hearing improvement (≥10 dB), 27 were unchanged, and 2 were worse at 125 Hz; 22 (47.8%) ears were improved, 23 unchanged, and 1 worse at 250 Hz; 18 (39.1%) ears were improved, 27 unchanged, and 1 worse at 500 Hz; 10 (21.7%) were improved, 33 unchanged, and 3 worse at 1000 Hz; 5 (10.9%) ears were improved, 39 unchanged, and 2 worse at 2000 Hz; 5 (10.9%) ears were improved, 40 unchanged, and 1 worse at 4000 Hz; 17 (37.0%) ears were improved, 27 unchanged, and 2 worse at 8000 Hz (Figure 7a).

The 'flat' group comprised 23 patients (4 males, 19 females) whose average age was 77.4 years (range 70–91 years). The average duration of treatment with radical scavengers during the study was 13.3 weeks (range 8–36 weeks). The treatment outcome regarding hearing was evaluated in 46 ears. The average pure-tone hearing level threshold in patients during pretreatment was 54.8±10.77 dB, i.e. differed significantly from the average thresholds after radical scavenger therapy: 48.9±10.29 dB at final observation ($p<0.001$). The final hearing levels had improved significantly compared with pretreatment at 125 Hz (53.2±12.49→44.0±13.52 dB;

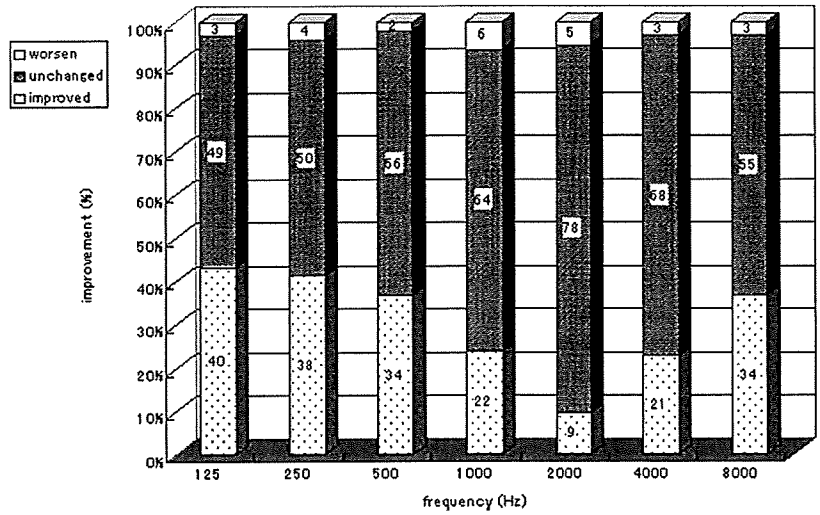

Figure 4. At the final follow-up, the proportion of clinically significant improvement in hearing was greatest at 125, 250, and 8000 Hz and lowest at 2000 Hz.

pretreatment→final) ($p<0.001$), 250 Hz ($52.0\pm14.92\rightarrow43.7\pm13.14$ dB) ($p<0.001$), 500 Hz ($48.3\pm13.22\rightarrow40.7\pm12.54$ dB) ($p<0.001$), 1000 Hz ($44.3\pm12.33\rightarrow40.7\pm12.45$ dB) ($p<0.01$), 4000 Hz ($58.2\pm14.92\rightarrow54.2\pm14.18$ dB) ($p<0.01$), and 8000 Hz ($79.8\pm17.82\rightarrow72.8\pm16.04$ dB) ($p<0.001$), but were unchanged at 2000 Hz ($48.0\pm12.22\rightarrow46.5\pm11.10$ dB) (Figure 6). There were no significant differences in hearing improvement between the 'steep' group and the 'flat' group at any frequency.

At the final follow-up, 23 (50.0%) ears showed a clinically significant hearing improvement (≥ 10 dB), 22 were unchanged, and 1 was worse at 125 Hz; 16 (34.8%) ears were improved, 27 unchanged, and 3 worse at 250 Hz; 16 (34.8%) ears were improved, 29 unchanged, and 1 worse at 500 Hz; 12 (26.1%) were improved, 31 unchanged, and 3 worse at 1000 Hz; 4 (8.7%) ears were improved, 39 unchanged, and 3 worse at 2000 Hz; 16 (34.8%) ears were improved, 28 unchanged, and 2 worse at 4000 Hz; 17 (37.0%) ears were improved, 28 unchanged, and 1 worse at 8000 Hz (Figure 7b).

Normal hearing ears vs impaired hearing ears

To evaluate the treatment outcome between the normal hearing group (better than mean age-corrected hearing level) and the impaired hearing group, treatment results in these two groups were compared. The two groups were divided according to the mean hearing level of the normal elderly population in Japan [17]. The average hearing level of those aged 70–74 years is 31.27 dB at 125 Hz, 31.91 dB at 250 Hz, 31.91 dB at 500 Hz, 34.79 dB at 1000 Hz, 42.41 dB at 2000 Hz, 55.01 dB at 4000 Hz and 67.60 dB at 8000 Hz; 35.11, 35.21, 35.55, 39.34, 46.84, 57.19, 71.55 dB, respectively, aged 75–75 years and 40.41, 41.01, 42.72, 45.20, 52.50, 64.88, 78.09 dB, respectively, aged over 80 years. The normal (appropriate to age) ears showed a significant improvement in hearing level at 125, 250, and 500 Hz after radical scavenger treatment, while impaired hearing ears showed a significant improvement at all frequencies. A significant difference in improved level was also noted at 125, 250, 500 1000, 4000, and 8000 Hz in the normal vs the impaired group (Table I, Figure 8).

Discussion

Age-related hearing loss is the primary cause of impaired hearing worldwide, implying a significant burden not only for sufferers, but also for those who communicate with them. The medical and socio-economic costs are immense, and given the increase in the world's population and the fact that the number of elderly individuals is expected to more than double by 2030, this problem is escalating [8].

In a number of studies, free radicals have been implicated in the damage associated with cochlear ischemia [18], noise trauma [5], aging, presbyacusis [8–11], and ototoxicity [6,7,20]. Biogerontologists have suggested possible ways to alleviate free radical-associated age-related changes (including presbyacusis). One way was to attempt to minimize the effects of mitochondrial damage, and to reduce free radical damage by dietary restriction and/or the use of dietary antioxidants [1]. Seideman and co-workers [8,9,18] also hypothesized that presbyscusis could be modulated by ingestion of dietary antioxidants (e.g. vitamins E and C, and antioxidant-rich

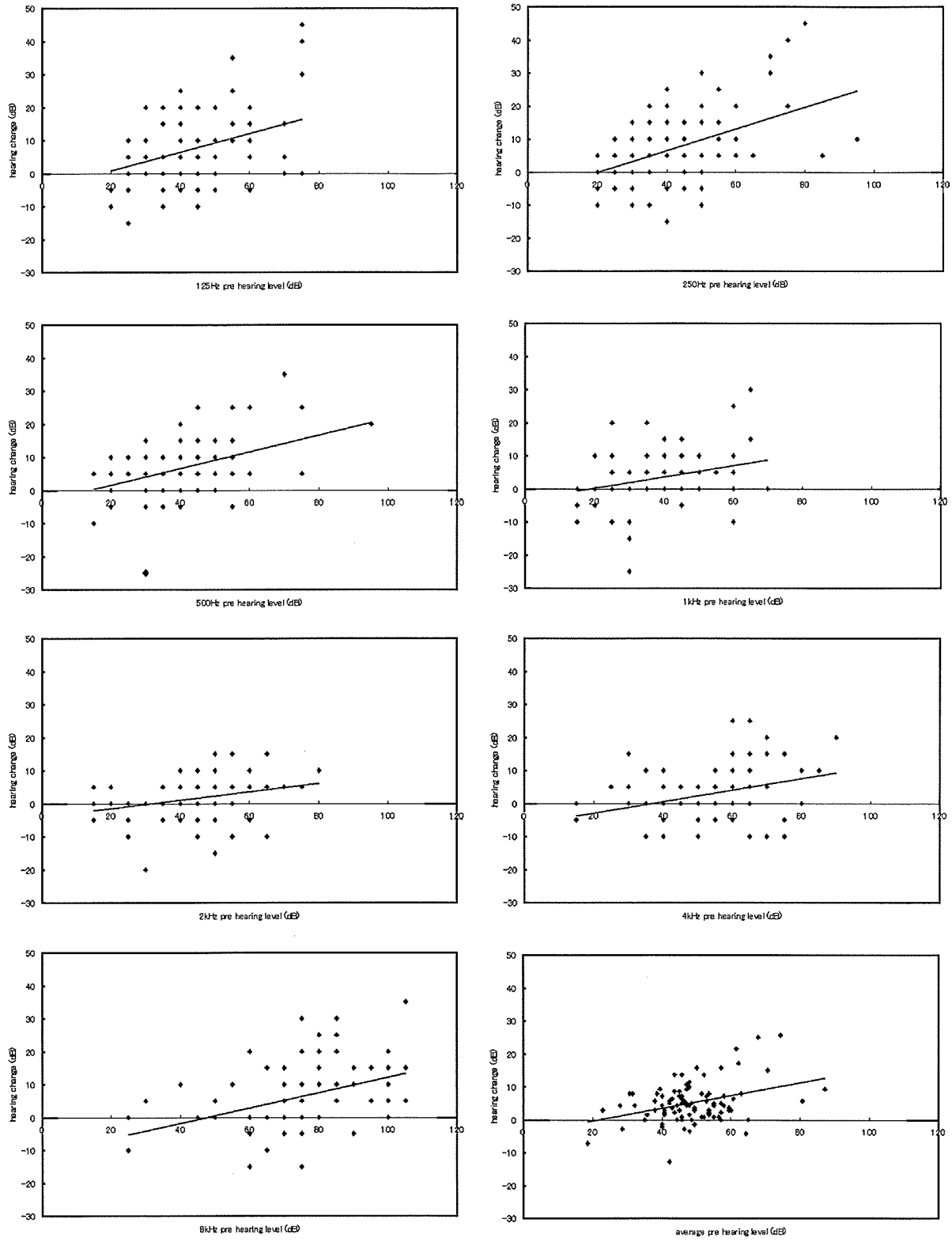

Figure 5. The correlation between pretreatment hearing level and change in hearing level was calculated for each ear at different frequencies. Significant correlations between pretreatment hearing levels and changes in hearing were noted at all frequencies.

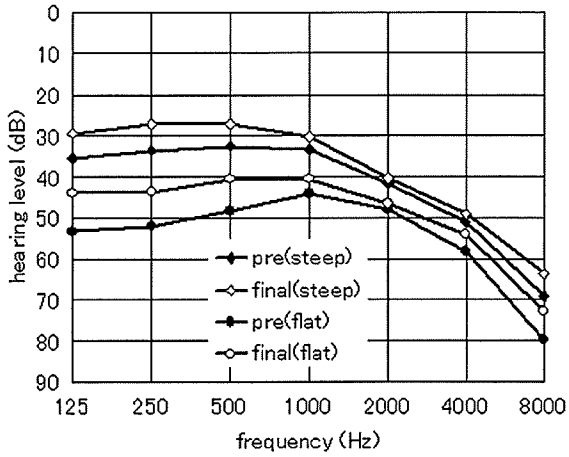

Figure 6. The final hearing levels were significantly improved compared with pretreatment, at all frequencies except 4000 Hz in the 'steep' group. In the 'flat' group, final hearing levels were significantly improved at all frequencies except 2000 Hz.

fruit and vegetables), as well as by dietary restriction. If dietary antioxidants – or any other nutritional supplement – have a beneficial effect on the efficiency of several human systems, it is conceivable that the functioning of the auditory system could also be improved, or at least maintained.

To apply radical scavengers for the treatment of age-related hearing loss, we selected rebamipide, α-lipoic acid, and vitamin C, as these drugs act as such and have already been widely used for other purposes, e.g. for the treatment of gastric ulcers and vitamin deficiency. These agents have also already been used clinically to treat cisplatin-induced hearing loss [13] and MD [12], and idiopathic sudden hearing loss [20], with satisfactory results [12].

The results of the present study demonstrated that some patients sustained a significant improvement in hearing, both subjectively and objectively, which would suggest the efficacy of this therapy. Radical

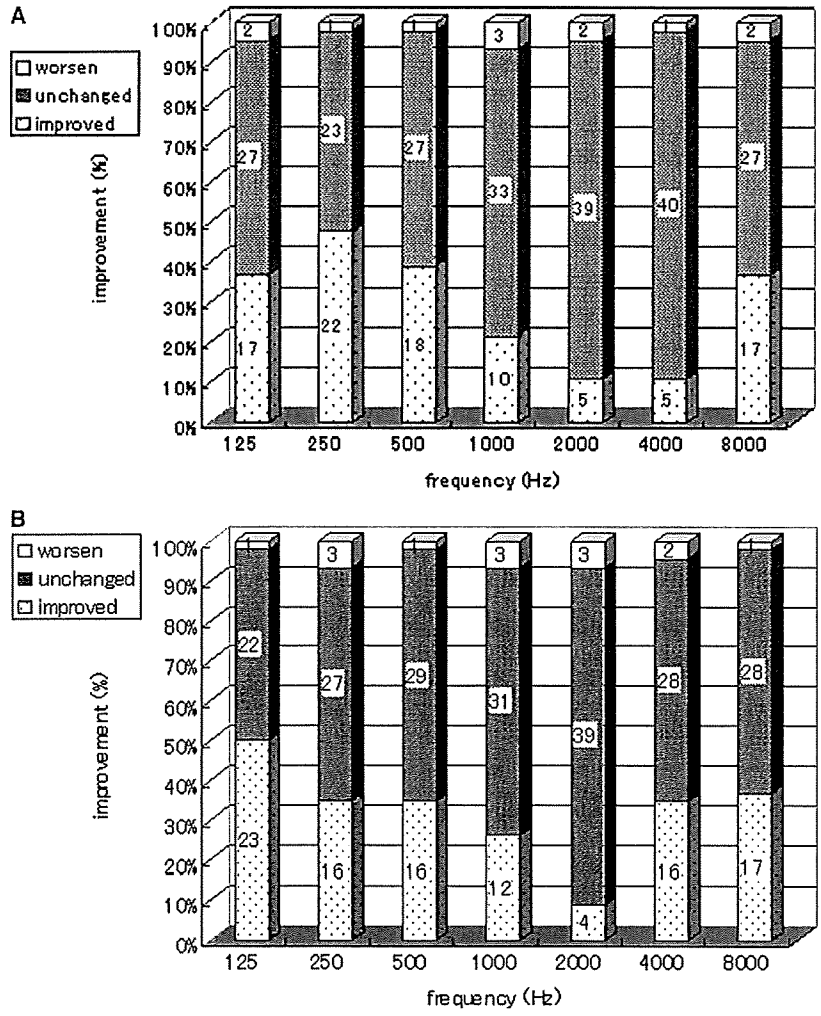

Figure 7. At the final follow-up, the proportion of clinically significant improvement in hearing in the 'steep' group was lower at 1000, 2000, and 4000 Hz (a). In the 'flat' group, improvement of hearing was lowest at 2000 Hz (b).

Table I. The average hearing level of the elderly population and the treatment results of both normal hearing ears and impaired hearing ears.

		Frquency (Hz)						
Parameter	Status	125	250	500	1000	2000	4000	8000
Mean hearing level (dB)	70–74 years	31.27	31.91	31.91	34.79	42.41	55.01	67.6
	75–79 years	35.11	36.21	35.55	39.34	46.84	57.19	71.66
	>80 years	40.41	41.01	42.72	45.2	52.5	64.88	78.09
Normal hearing (dB)	Pretreatment	31.4 ($n=32$)	29.4 ($n=34$)	29.3 ($n=38$)	29 ($n=46$)	36.6 ($n=48$)	44.1 ($n=46$)	60 ($n=38$)
	Post treatment	27.3	26.3	25.4	27.4	36	43.6	58.3
		$p<0.05$	$p<0.05$	$p<0.01$	NS	NS	NS	NS
Impaired hearing (dB)	Pretreatment	51.1 ($n=60$)	50.6 ($n=58$)	48.2 ($n=54$)	48.7 ($n=46$)	54.2 ($n=44$)	65.3 ($n=46$)	85.3 ($n=54$)
	Post treatment	41.7	40.8	39.8	43.5	51.4	59.9	75.7
		$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.01$	$p<0.001$	$p<0.001$
Normal vs impaired		$p<0.05$	$p<0.01$	$p<0.05$	$p<0.05$	NS	$p<0.01$	$p<0.001$

scavenger therapy produced significant hearing improvement at all frequencies.

Concerning the etiology of ARHL, various types of pathological changes, namely sensory, neural, strial, cochlear conductive, mixed, and intermediate, are known [2,21]. Changes in sensory functioning are generally associated with a steep high-frequency hearing loss but usually well preserved speech recognition ability. The main histopathological changes occur in the organ of Corti, with loss of sensory and supporting cells. Sensory cell degeneration occurs typically at the extreme basal end of the cochlea; the outer hair cells degenerate first, followed by inner hair cells. Improvement in hearing at high frequencies (especially at 8000 Hz) may be due to the improvement of sensory cell function, as it has been reported that the formation of free radicals in the sensory cells may be a crucial factor in such forms of hearing loss as acoustic trauma, labyrinthitis, aminoglycoside ototoxicity, cisplatin ototoxicity, MD, and presbyacusis [3–7]. It has also been demonstrated that radical scavengers provide protection from inner ear damage caused by aminoglycosides, cisplatin, lipopolysaccharide-induced labyrinthitis, acoustic trauma, and presbyacusis [3–7].

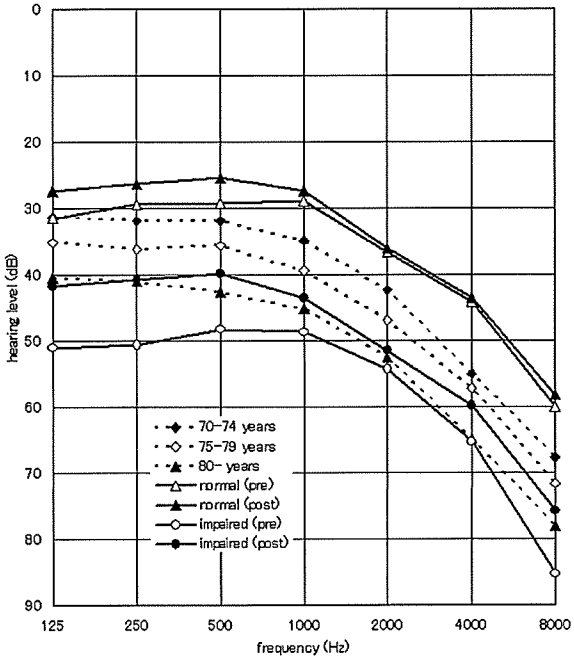

Figure 8. A normal-hearing ear showing significant improvement in hearing at low frequencies (125, 250, and 500 Hz). Impaired hearing ears showed significant improvement at low frequencies [17].

Improved hearing at low frequencies may result from improved functioning of the stria vascularis, in which free radical formation under pathological conditions has also been noted [4,7,19]. The stria vascularis appears to be an ion transport and control structure. It produces the necessary ion concentrations in the endolymph that enable generation of the endocochlear potential, an essential process for normal cochlear functioning. Atrophy of stria vascularis, including loss of both strial tissue and cells, chiefly in the apical and middle turns of the cochlea, is characteristic of age-related strial hearing loss. The loss of strial tissue in aging ears is believed to affect some endolymph characteristics, which in turn negatively affect the physical and chemical processes by which energy essential for cochlear functioning is provided. Although audiograms associated with age-related strial hearing loss are not uniform, findings typically include a slowly progressing, symmetrical,

relatively flat, mild-to-moderate hearing loss, but good speech recognition [2,21].

In our earlier, pilot study [15], we reported significant threshold improvements at 125, 250, 500, and 8000 Hz, but no significant improvement at 1000, 2000, or 4000 Hz. In the present investigation, the improvement of hearing was also lower at 1000, 2000, and 4000 Hz, but significant at all frequencies. Moreover, some patients did show marked improvements at 1000, 2000, and 4000 Hz as well. This was also the case in patients with MD [12] and cisplatin ototoxicity [13]. In animal experiments, it has been suggested that inner ear sensory cells are capable of sustaining sublethal damage, although it is virtually impossible to recover function after complete cell death [22]. This is also the case in human subjects.

In the present study, to avoid all causes of hearing loss other than presbyacusis, we selected the patients more strictly and increased their number. In addition, analysis was also made between the patients with a 'steep'-type audiogram and the patients with a 'flat' audiogram. 'Steep' audiogram may represent sensory or neural presbyacusis and 'flat' audiogram may represent strial presbyacusis. The 'steep' group showed a significant improvement in hearing at all frequencies except 4000 Hz, while the 'flat' type showed a significant improvement at all frequencies except 2000 Hz. Moreover, there were no significant differences in the improvement of hearing at all frequencies between these two groups. These findings may corroborate the efficacy of radical scavengers for the treatment of any type of presbyacusis.

Concerning the limitation of this therapy, we studied the difference between normal hearing (appropriate to age) ears and impaired hearing ears. The former showed significant improvement only at low frequencies. Similar results were obtained in a double-blind, randomized, placebo-controlled trial of folic acid supplementation on hearing in older adults. After 3 years of folic acid supplementation, thresholds in the low frequencies rose by 1.0 dB and by 1.7 dB in the placebo groups ($p = 0.020$) [23]. Folic acid supplementation did not arrest the deterioration of hearing at high frequencies. This could be the limitation of this form of therapy. The present results also showed a positive correlation between pretreatment hearing levels and improvement in hearing at all frequencies, i.e. the ear with poorer hearing has a greater possibility to recover. Impaired hearing ears showed better treatment results than normal-hearing ears, but did not surpass normal-hearing levels. In other words, radical scavenger therapy can improve impaired hearing to the normal (mean) hearing level appropriate to age, but not to a better hearing level of younger age. This is the limitation of the treatment.

Regarding the application of radical scavengers to presbyacusis, there are two ways; one is for prevention, the other is for treatment. In animal studies, radical scavengers were used for both prevention and treatment. In a rat model, a 30 day treatment of 24-month-old rats with 50 mg/kg l-carnitine significantly ameliorated age-related deterioration of auditory pathways, thus demonstrating the efficacy of this therapy for treatment of presbyacusis [10]. In a dog model, an antioxidant diet (α-tocopherol, l-carnitine, α-lipoic acid, ascorbic acid) for 3 years showed reduced degeneration of spiral ganglion cells and stria vascularis vs the control diet group. Treatment with vitamins C, E, or melatonin gave protection from ARHL in rats [8,9]. These studies showed the efficacy of radical scavengers for prevention of presbyacusis [11]. The present study represents the treatment of presbyacusis. However, it does have one limitation: the treatment could not give any improvement beyond the normal hearing level appropriate to age. This may suggest that prevention of presbyacusis is more important, which is the next step. The antioxidant diet and/or supplementation with radical scavengers at a younger age might be a suitable way to prevent presbyacusis.

In this study, we chose rebamipide, vitamin C, and α-lipoic acid as radical scavengers. Besides these drugs, there are several other candidates, i.e. vitamin E (α-tocopherol) [8,14,20], l-carnitine [8], ebselen [24], edaravone [25], etc. All of these were confirmed as capable of reducing inner ear damage in an animal model. In addition, vitamin E [14] – or a combination of vitamins B and E [20] – has been used to treat idiopathic sudden hearing loss, with acceptable results. Further evaluation of this new drug combination may be useful for the treatment of presbyacusis.

In conclusion, the results of this study demonstrated that treatment with radical scavengers has the potential to become an effective new therapy for presbyacusis. However, this finding is only preliminary in view of the short follow-up period. Further evaluation of this treatment modality for recovery from or prevention of hearing loss is necessary. In addition, a closely controlled randomized study needs to be performed to confirm the efficacy of this treatment.

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ORIGINAL ARTICLE

Changes in transient receptor potential vanilloid (TRPV) 1, 2, 3 and 4 expression in mouse inner ear following gentamicin challenge

TAKUYA ISHIBASHI[1], MASAYA TAKUMIDA[1], NANA AKAGI[2], KATSUHIRO HIRAKAWA[1] & MATTI ANNIKO[3]

[1]*Department of Otolaryngology, Hiroshima University Faculty of Medicine,* [2]*Hiroshima University School of Medicine, Hiroshima, Japan and* [3]*Department of Otolaryngology, Head and Neck Surgery, University Hospital, Uppsala, Sweden*

Abstract

Conclusion. It is suggested that transient receptor potential vanilloid (TRPV)-1 and -2 may be of pathological significance for sensory cells and ganglions, while TRPV-3 and -4 may play an important part in neuroprotection of the inner ear. *Objective.* Changes in the expression of TRPV-1, -2, -3, and -4 in gentamicin (GM)-treated mouse inner ear were studied. *Materials and methods.* CBA/J mice were used in this study. The localization of TRPV-1, -2, -3, and -4 in the inner ear of both untreated and GM-treated CBA/J animals (intratympanic injection of 5 mg GM) was investigated by immunohistochemistry. *Results.* TRPV-1, -2, and -3 were co-expressed in the inner ear sensory and ganglion cells, while TRPV-4 was also expressed in the stria vascularis and vestibular dark cells. Following GM treatment, the intensity of immunofluorescent reaction to TRPV-1 and TRPV-2 increased, while that to TRPV-3 and TRPV-4 decreased.

Keywords: *TRPV, inner ear, mouse, gentamicin, immunohistochemistry*

Introduction

Vertebrate transient receptor potential vanilloid (TRPV) channels are known to be sensitive to many forms of physical and chemical stimuli. All types of vertebrate TRPV are calcium-permeable channels, of which the TRPV-1, -2, -3, and -4 groups are characterized as moderately calcium selective cation channels and TRPV-5 and TRPV-6 are highly selective calcium channels. There is increasing evidence that the TRPV-1–4 isoforms are sensitive to both noxious and innocuous physical stimuli, ranging from heat to osmolality/stretch, to shear stress/flow, and possibly to pressure. TRPV-1 is expressed in dorsal root ganglia (DRG), central nervous system (CNS), urinary bladder, and vessels and is activated by capsaicin, protons, heat (>42°C), anandamide, etc. The physiological roles of TRPV-1 are thermal pain sensation, mechanosensation, vascular regulation, and taste transduction. TRPV-2 is abundantly expressed in DRG, CNS, lung, spleen, small and large intestines, endothelial cells, and vascular smooth muscles. TRPV-2 is activated by injurious heat (>52°C), cell distension, and mechanical pressure. Its physiological roles are thermosensation and mechanosensation. TRPV-3 is expressed in DRG, skin, testis, stomach, small intestine, trachea, placenta, and keratinocytes, and it is activated by temperature (>31°C) and diphenylboronic anhydride. Its physiological roles are not fully understood, but may be related to thermosensation (thermal preference) and algesia. TRPV-4 is expressed in DRG, kidney, skin, inner ear, endothelium, brain, hypothalamus, trachea, lung, fat, and heart. Its physiological roles are osmotic regulation by the CNS, mechanically and osmotically mediated algesia, and thermal preference (>27°C) [1].

We have already demonstrated the presence of TRPV-1, -2, -3, and -4 in the inner ear. TRPV-1 was identified in the organ of Corti and inner ear ganglion cells, while TRPV-4 was found in both inner (IHCs) and outer hair cells (OHCs) of the organ of Corti, in spiral ganglion cells, marginal cells of stria vascularis, sensory cells in the vestibular end

Correspondence: Takuya Ishibashi MD, Department of Otolaryngology, Hiroshima University Faculty of Medicine, 1-2-3 Kasumicho, Minamiku, Hiroshima 734-8551, Japan. E-mail: d055860@hiroshima-u.ac.jp

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organs, vestibular ganglion cells, and in the endolymphatic sac [2]. More recently, TRPV-2 and TRPV-3 have also been identified in the inner ear and often co-localized with TRPV1. TRPVs are believed to play a functional role in sensory cell physiology. TRPV-4 and TRPV-2 in particular may be important for fluid homeostasis in the inner ear [2]. However, the relationship between the pathological conditions and the changes in TRPV expression remains obscure.

It is well known that the ototoxic effects of aminoglycosides are attributable, at least partially, to stimulation of free radical formation in inner ear tissues [3,4]. Brain-derived neurotrophic factor (BDNF) becomes up-regulated after gentamicin (GM) treatment. The formation of free radicals and neurotrophins is known to activate certain TRPVs [1]. In pathological conditions, changes in TRPV expression occur in DRG and trigeminal ganglia (TG) [1,5]. Some members of the TRPV family are important mediators of nociception and hyperalgesia. Other studies suggest that TRPV1 expression is regulated dynamically as a component of responses to potentially injurious challenges. Based on the finding of TRPVs and concomitant pathological conditions of the inner ear, it has been suggested that the expression of TRPVs in the inner ear must also change under pathological conditions.

The purpose of the present study was to investigate changes in TRPV expression in the inner ear following GM challenge with regard to new pathophysiological mechanisms where the TRPVs become involved.

Materials and methods

We used nine healthy, otomicroscopically normal, 8-week-old CBA/J mice with body weights in the range 20–25 g and a normal Preyer's reflex. Care and use of the animals was approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. A06-149) and was in accordance with the Guide to Animal Experimentation, Hiroshima University and the guidelines of the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine.

The animals were randomly divided into a control group and a GM-treated group. Six animals in the latter group were injected with a single 25 µl (5 mg) dose of GM solution (200 mg GM per 1 ml sterile saline; Sigma Chemical Co., St Louis, MO, USA) through the tympanic membrane into the tympanic bulla of the left ear. For control purposes, the same volume of saline was injected into the tympanic bulla of three control animals. Two weeks after the injections, all animals were deeply anesthetized with pentobarbital and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. The temporal bones were excised and immersed in the same fixative for a further 1 h. They were decalcified with 0.1 M buffered Na-EDTA for 14 days. All specimens were frozen in OCT mounting medium (Sakura Finetechnical Co. Ltd, Tokyo, Japan), serially sectioned on a cryostat at 4 µm, and mounted on glass slides. After pretreatment with blocking serum, the specimens were incubated with a rabbit polyclonal antibody to TRPV-1 (Transgenic Inc., Kumamoto, Japan) at a dilution of 0.1 µg/ml, rabbit polyclonal antibody to TRPV-2 (Abcam, Tokyo, Japan) diluted 1:1000, goat polyclonal antibody to TRPV-3 (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:50, a rabbit polyclonal antibody to TRPV-4 (Alomone Labs Ltd, Jerusalem, Israel) diluted 1:200, a rabbit polyclonal antibody to hydroxynonenal (Alexis Biochem, Lausanne, Switzerland) diluted 1:500, a rabbit polyclonal antibody to nitrotyrosine (Upstate, NT, USA) diluted 10 µg/ml, or with a mouse monoclonal antibody to BDNF (R&D systems, MN, USA) diluted 1:40 in 0.3% Triton X-100 containing phosphate-buffered saline (PBS) at 4°C for 48 h. The specimens were then washed in PBS and incubated for 1 h with Alexa Fluor 488 goat anti-rabbit, donkey anti-goat, or rabbit anti-mouse secondary antibodies (1:500) (Molecular Probes, Eugene, OR, USA). The sections were washed and cover-slipped with DakoCytomatia Fluorescent Mounting Medium (DakoCytomatia, CA, USA).

For the double-staining studies, the sections were first stained with TRPV-3 followed by secondary antibodies conjugated with Alexa 568 and then stained for TRPV-1, -2, and –4, with the secondary antibodies conjugated with Alexa 488. The specimens were viewed in a Nikon fluorescence microscope (Eclipse E600) equipped with an appropriate filter set. Fluorescence analog images were obtained via an intensified digital color charge-coupled device camera (C4742-95; Hamamatsu Photonics) and stored as digital images using IP Lab Spectrum software (version 3.0; Signal Analytics Corporation).

Measurements of fluorescence intensities

For the statistical analysis, we measured the fluorescence intensity of the different parts, i.e. stria vascularis, OHCs, vestibular sensory cells, spiral ganglion cells, and vestibular ganglion cells. Ten sensory cells, 10 ganglion cells, and 10 cells in the

stria vascularis were randomly selected from each specimen, and their fluorescence intensity was measured. The value of the 10 cells was averaged for each specimen. The grand mean was obtained by averaging the means of six to eight specimens for each group. A standard error was calculated from the means for individual specimens. These data were analyzed by a two-way analysis of variance (ANOVA). To visualize fluorescence intensity, some specimens were depicted in pseudocolor; purple represented intense fluorescence and red represented weak fluorescence, using IP Lab Spectrum software.

Results

Distribution of TRPVs in untreated mouse inner ear

In the normal mouse, TRPVs are expressed in the inner ear (as already reported in our previous paper). Briefly, TRPV-1 labelling was observed chiefly in IHCs and OHCs, in some supporting cells and in spiral ganglion cells, whereas stria vascularis did not display any significant fluorescence (Figure 1a). Evidence of TRPV-1 expression was also found in the vestibular sensory cells of crista ampullares, in both the utricular and saccular maculae, as well as in the vestibular ganglion cells. In the subepithelial

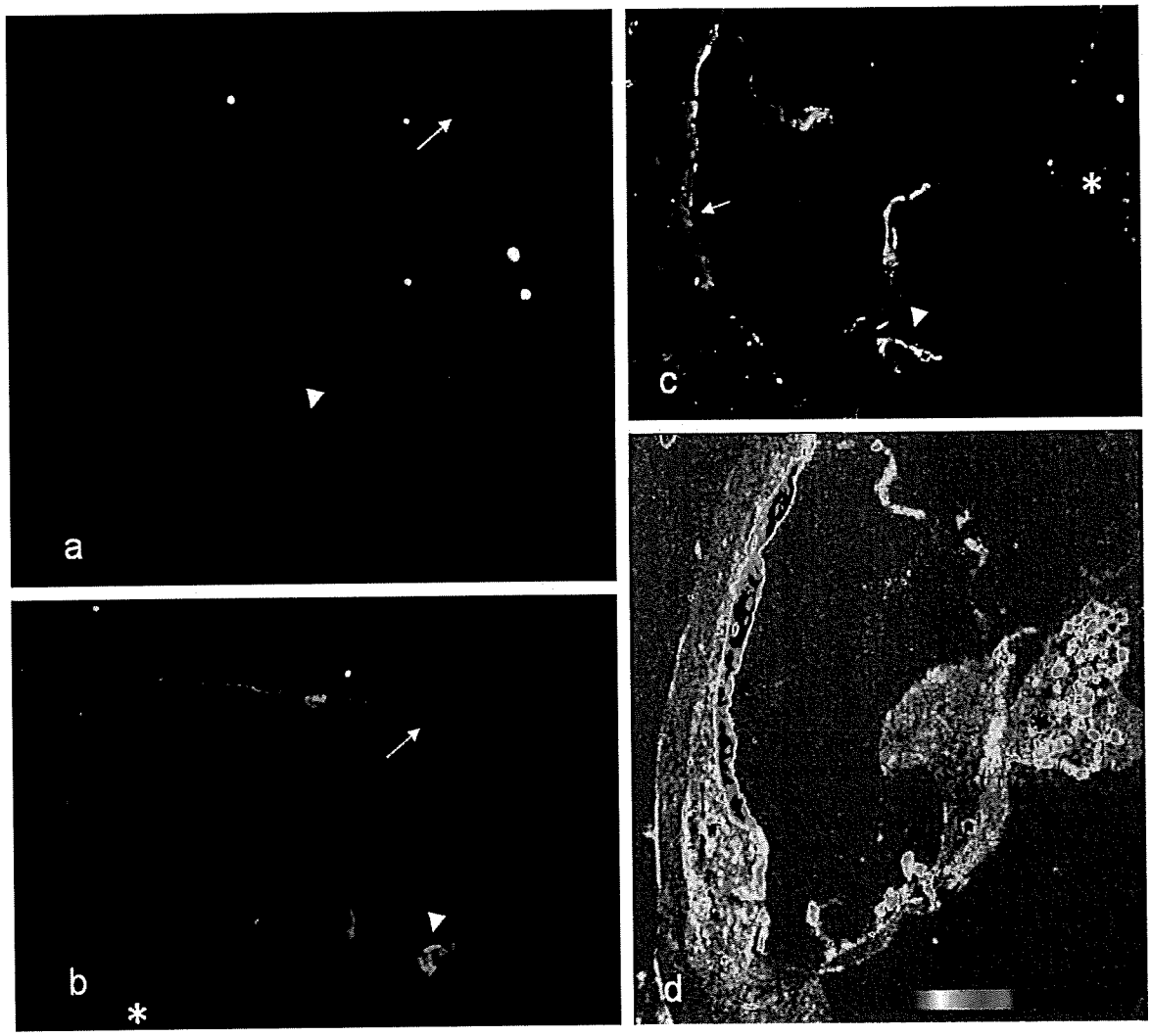

Figure 1. (a) In mouse cochlea, TRPV-1 labelling is evident in hair cells (arrowhead), while stria vascularis shows no significant fluorescence (arrow). (b) Cochlear hair cells (arrowhead) and spiral ganglion cells (asterisk) show a positive immunofluorescence for TRPV-2. In stria vascularis, TRPV-2 shows weak fluorescence (arrow). (c) The double-staining study for TRPV-3 (red) and TRPV-4 (green) revealed yellow fluorescence denoting co-expression of TRPV-3 and TRPV-4 (yellow) in the organ of Corti (arrowhead) and spiral ganglion cells (asterisk), while stria vascularis revealed only green fluorescence, denoting TRPV-4 immunoreactivity (arrow). (d) Pseudocolor image of cochlear TRPV-4. Fluorescence intensity for TRPV-4 is strongest in stria vascularis (arrow). Spiral ganglion cells (asterisk) and hair cells (arrowhead) also show some immunoreactivity.

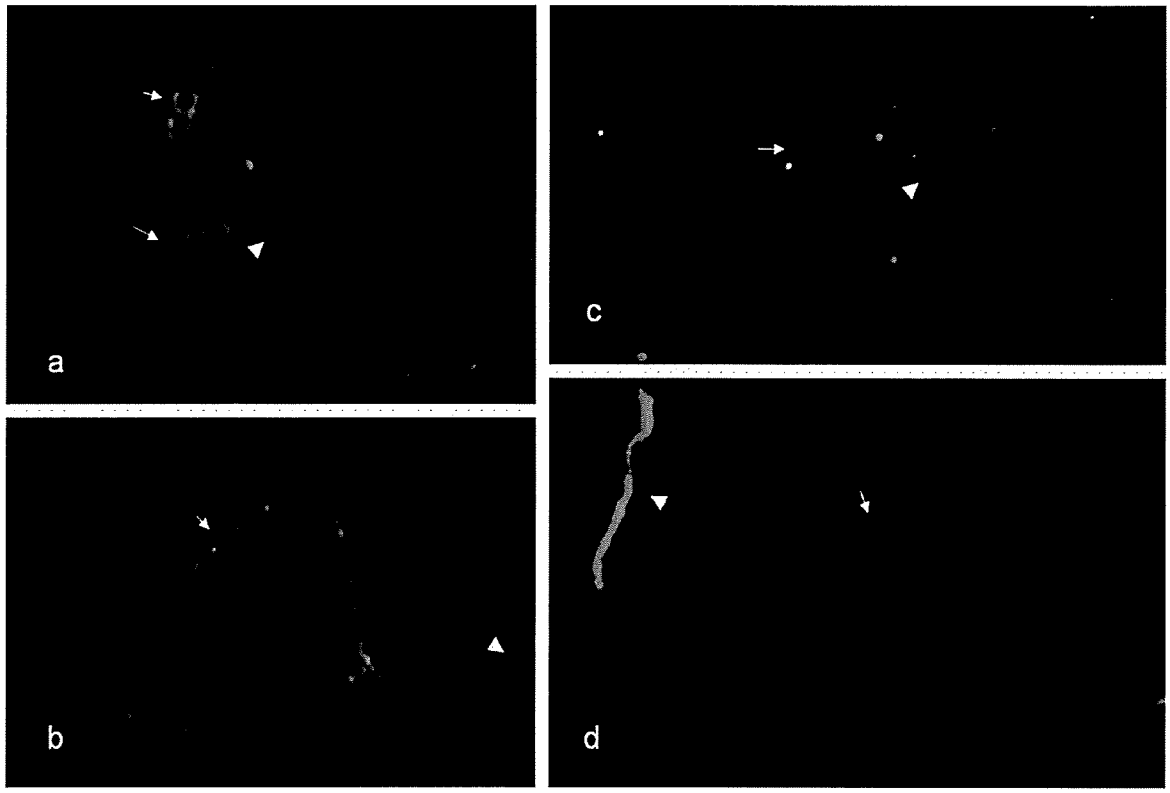

Figure 2. (a) In the vestibular epithelia of crista ampullares, immunoreactivity to TRPV-1 is evident in sensory cells (arrows). In the subepithelial tissue, nerve fibers connected to the sensory cells show moderate fluorescence (arrowhead). (b) Immunoreactivity to TRPV-2 is evident in sensory cells (arrow). The dark cells shown are only weakly immunofluorescent (arrowhead). (c) Immunoreactivity to TRPV-3 is visible in the sensory cells (arrow). In the subepithelial tissue, nerve fibers connected to the sensory cells show moderate fluorescence (arrowhead). (d) Immunoreactivity to TRPV-4 is visible in the sensory cells (arrow). The dark cells show marked immunoreactivity (arrowhead).

tissue, nerve fibers connected to the sensory cells were moderately fluorescent (Figure 2a). TRPV-2 was also expressed in the sensory cells of the organ of Corti and in vestibular sensory cells, and spiral and vestibular ganglion cells (Figures 1b and 2b). In stria vascularis and vestibular dark cells, TRPV-2 was weakly fluorescent, especially in the apical cytoplasm of the marginal cells (Figure 1b). The distribution of TRPV-3 was almost identical with that of TRPV-1 (Figures 1c and 2c). TRPV-4 labeling was evident in OHCs, with comparatively weak labeling in IHCs. The region of stria vascularis (especially marginal cells) displayed marked fluorescence, while the spiral prominence showed only faint fluorescence. TRPV-4 expression was also observed in the spiral ganglion cells (Figure 1c). In the cochlea, fluorescence intensity was greatest in stria vascularis (Figure 1d). In the vestibular end organs, immunoreactivity to TRPV-4 was observed in both sensory cells and ganglion cells. The dark cells displayed marked immunoreactivity, while transitional cells showed only weak labelling (Figure 2d).

Immunohistochemistry for hydroxynonenal, nitrotyrosine, and BDNF

In the normal mouse inner ear, weak immunoreactivity to hydroxynonenal and nitrotyrosine was observed in the hair cells of the organ of Corti, stria vascularis, and spiral ganglion cells (Figure 3a, c). In the vestibular organs, immunoreactivity was weak in both sensory cells and vestibular ganglion cells. Immunoreactivity to BDNF was similar, although the fluorescence intensity was generally weaker (Figure 3e); in particular, stria vascularis showed only faint immunoreactivity (Figure 4a). In the GM-treated animals, immunoreactivity to hydroxynonenal, nitrotyrosine, and BDNF became more intense, especially in the spiral and vestibular ganglion cells (Figure 3 and Figure 4a, b).

Changes in expression of TRPVs in GM-treated mouse inner ear

The expression patterns of TRPVs in the inner ears of GM-treated mice were quite similar to those in

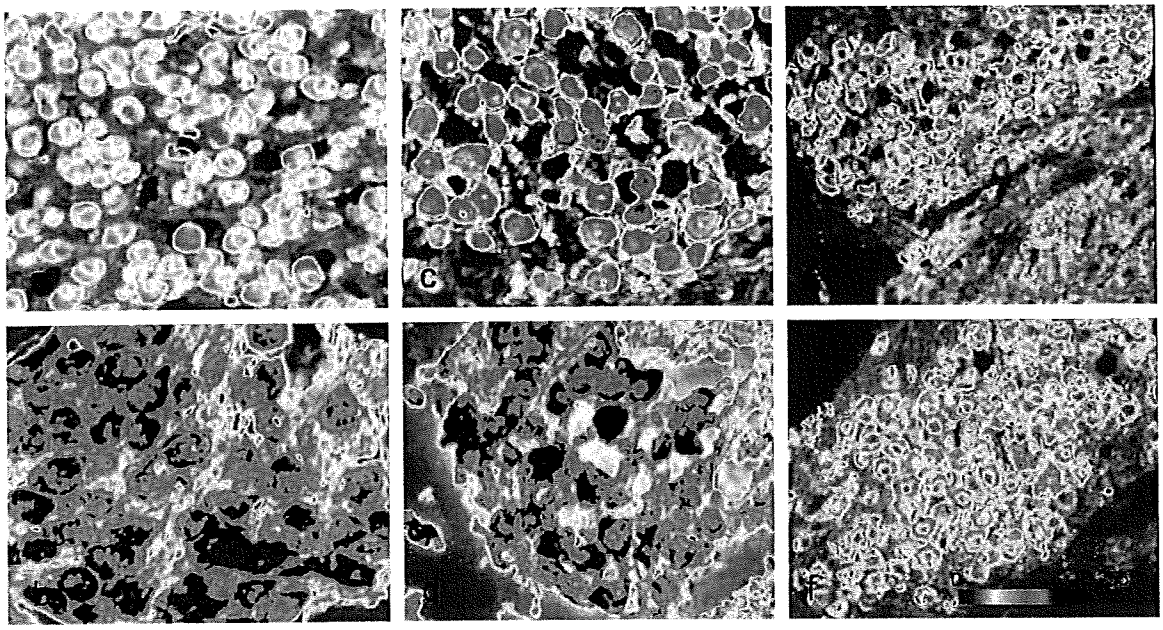

Figure 3. Pseudocolor images of immunoreactivity to hydroxynonenal (a, b), nitrotyrosine (c, d), and BDNF (e, f) in spiral ganglion. Spiral ganglion cells show immunoreactivity for hydroxynonenal (a), nitrotyrosine (c), and BDNF (e) in control animals. Fluorescence is intensified after GM treatment (b, d, f).

untreated mice, but fluorescence intensity changed following GM treatment. The fluorescence of TRPV-1 and TRPV-2 intensified in every part, i.e. cochlear hair cells, spiral ganglion cells, vestibular sensory cells, vestibular ganglion cells (Figure 5a, b and Figure 6), but weakened generally in TRPV-3 and TRPV-4 (Figure 5c, d and Figure 7). In stria vascularis and the vestibular dark cells, the intensity

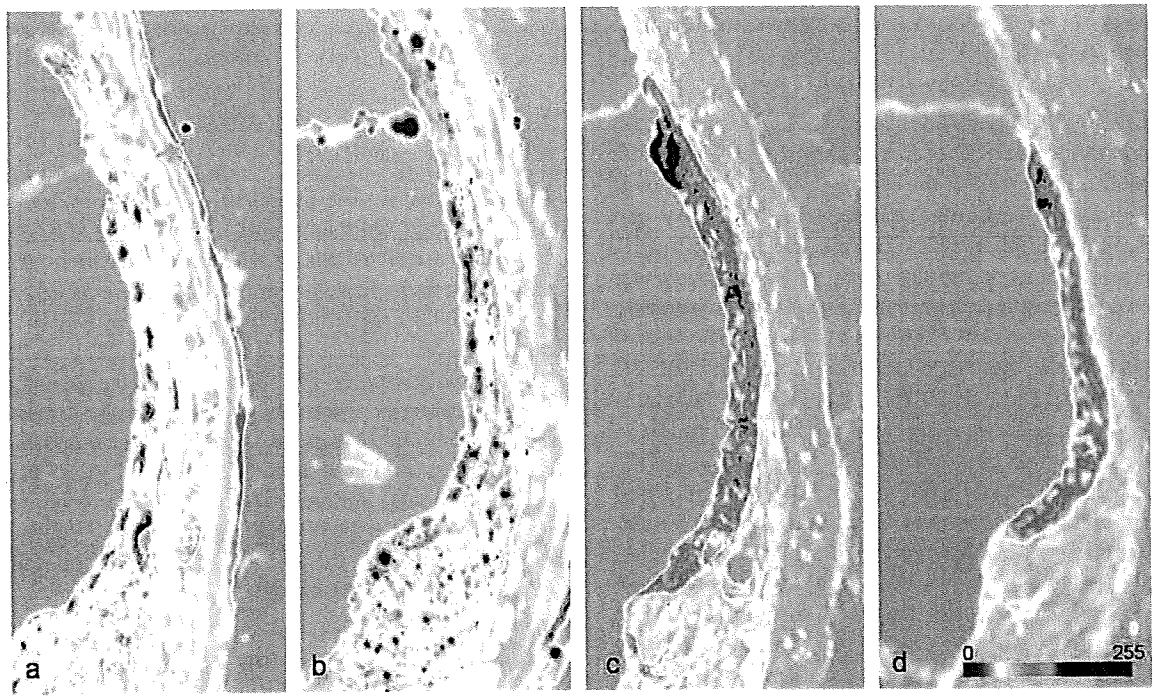

Figure 4. Pseudocolor images of immunoreactivity to BDNF (a, b) and TRPV-4 (c, d) in stria vascularis. Weak immunoreactivity to BDNF is observed in controls (a). Fluorescence intensity is increased after GM treatment (b). TRPV-4 is expressed in normal stria vascularis (c), but reduced following GM treatment (d).

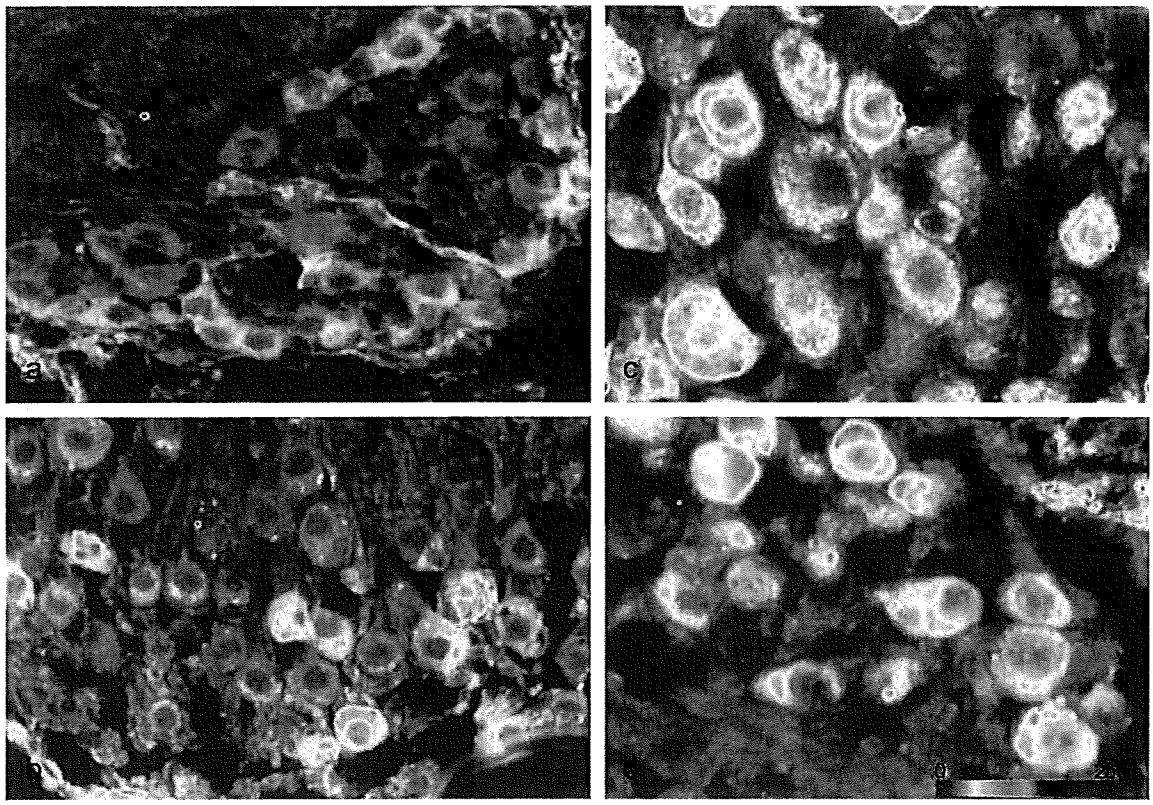

Figure 5. Pseudocolor images of immunoreactivity to TRPV-1 (a, b) and TRPV-4 (c, d) in vestibular ganglia. TRPV-1 expression is evident in vestibular ganglion cells (a), and increased following GM treatment (b). TRPV-4 is also noted in vestibular ganglion cells (c), but is reduced following GM treatment (d).

of fluorescent reaction to TRPV-4 also abated (Figure 4c, d).

In order to explain these changes in fluorescence intensity, changing ratios (%) were calculated as follows. Fluorescence change ratio (%) = grand mean of the intensity in each part of the GM-treated animals/grand mean of the intensity in the corresponding part of the untreated mice.

The fluorescence intensity changes of BDNF were $184 \pm 36.1\%$ ($p < 0.01$) in OHCs, $205 \pm 30.3\%$ ($p < 0.01$) in spiral ganglions, $136 \pm 14.1\%$ ($p < 0.01$) in stria vascularis, $138 \pm 7.2\%$ ($p < 0.05$) in vestibular sensory cells, and $129 \pm 67.9\%$ ($p < 0.01$) in vestibular ganglion cells in the GM-treated animals.

The fluorescence intensity changes of TRPV-1 were $117 \pm 7.5\%$ ($p < 0.05$) in OHCs, $177 \pm 47.8\%$ ($p < 0.01$) in spiral ganglions, $125 \pm 5.3\%$ ($p < 0.01$) in vestibular sensory cells, and $154 \pm 60.3\%$ ($p < 0.01$) in vestibular ganglion cells of GM-treated animals.

The fluorescence intensity changes of TRPV-2 were $113 \pm 4.3\%$ ($p < 0.05$) in OHCs, $119 \pm 14.6\%$ ($p < 0.05$) in spiral ganglions, $119 \pm 4.6\%$ ($p < 0.05$) in vestibular sensory cells, and $121 \pm 20.8\%$ ($p < 0.05$) in vestibular ganglion cells of GM-treated animals.

The fluorescence intensity changes of TRPV-3 were $77 \pm 11.5\%$ ($p < 0.05$) in OHCs, $57 \pm 9.2\%$ ($p < 0.01$) in spiral ganglions, $80 \pm 4.2\%$ ($p < 0.01$) in vestibular sensory cells, and $71 \pm 24.3\%$ ($p < 0.05$) in vestibular ganglion cells of GM-treated animals.

The fluorescence intensity changes of TRPV-4 were $65 \pm 16.3\%$ ($p < 0.01$) in OHCs, $49 \pm 22.1\%$ ($p < 0.01$) in spiral ganglions, $68 \pm 11.4\%$ ($p < 0.05$) in stria vascularis, $89 \pm 7.6\%$ ($p < 0.05$) in vestibular sensory cells, and $73 \pm 25.6\%$ ($p < 0.01$) in vestibular ganglion cells of GM-treated animals (Figure 8).

Discussion

The present study demonstrated that all four TRPV families are expressed in the mouse inner ear. Intratympanic GM treatment intensified the fluorescent reaction to TRPV-1 and TRPV-2, but decreased it for TRPV-3 and TRPV-4. These results indicate an up-regulation of TRPV-1 and TRPV-2 and a down-regulation of TRPV-3 and TRPV-4 in the inner ear following GM treatment.

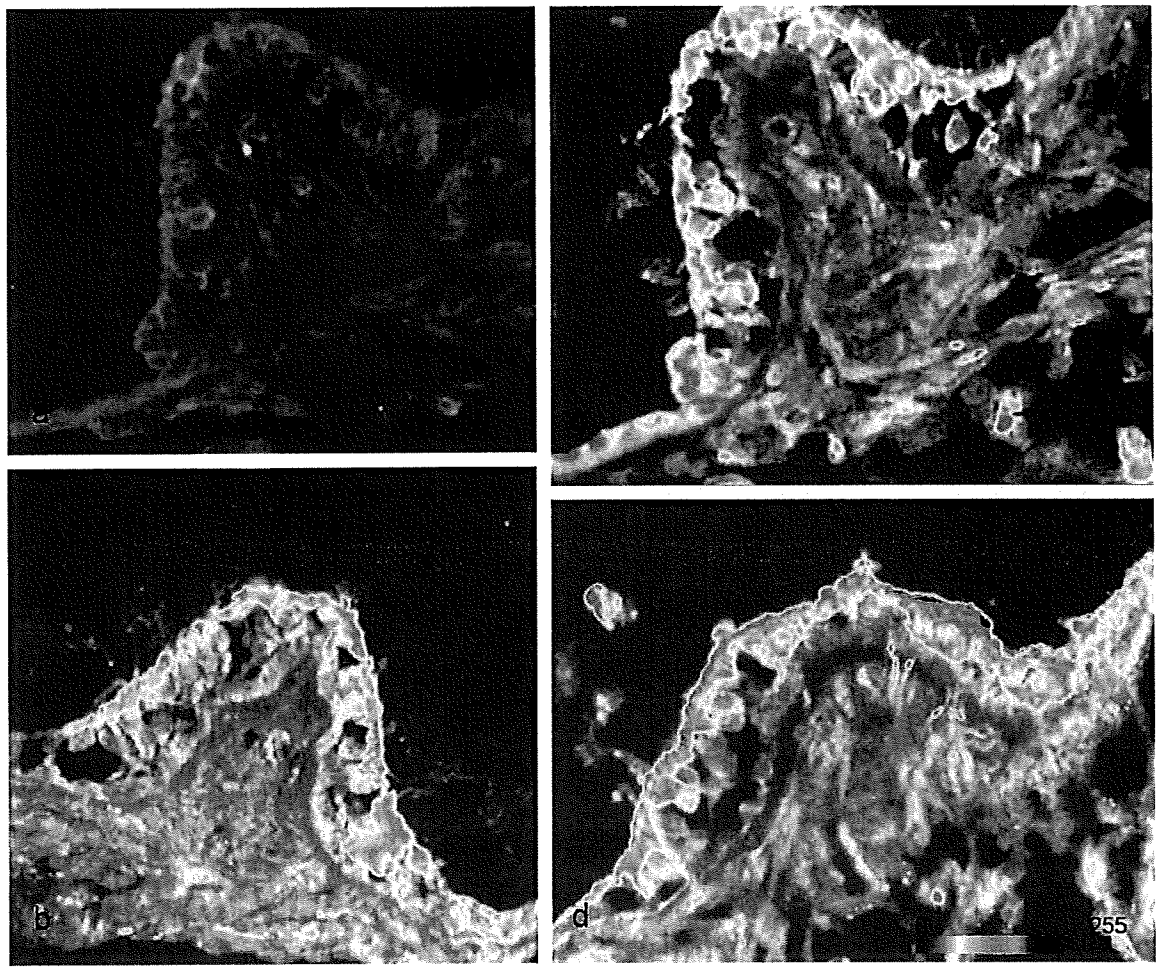

Figure 6. Pseudocolor images of immunoreactivity to TRPV-1 (a, b) and TRPV2 (c, d) in crista ampullaris. (a) TRPV-1 (a) and TRPV-2 (c) labeling is visible in sensory cells and nerve fibers. Fluorescence intensity is increased following GM treatment (b, d).

An up-regulation of TRPV-1 was noted in the cochlear and vestibular sensory cells and in the spiral and vestibular ganglion cells. Kanamycin (KM) treatment produced a seven- to eight-fold up-regulation of TRPV-1 mRNA in both inner ear ganglia, but that was attenuated by concurrent antioxidant treatment. This up-regulation of TRPV-1 mRNA to KM treatment did not occur in the TG or kidney. These mRNA changes were paralleled by a five- to six-fold up-regulation of TRPV-1 protein expression in the inner ear ganglia [2]. Following exposure to noise, TRPV-1 immunoreactivity increased in all cochlear regions between 24 h and 2 weeks afterwards. At the longest survival interval (16.9 months), TRPV-1 density was dramatically reduced in the basal region. Psychophysical testing of the long-survival animals revealed evidence of 20 kHz tonal tinnitus [6]. These results suggested that TRPV-1 may participate after cochlear injury in a single cascade that is responsible for the neuropathic events leading to tinnitus and hyperacusis.

In pathological conditions, an up-regulation of TRPV-1 during inflammation in DRG cells has been presented. Several studies have demonstrated that inflammatory mediators (bradykinin, prostaglandin E_2, extracellular ATP, glutamate, and nerve growth factor) indirectly sensitize TRPV-1. Following exposure of sensory neurons to inflammatory mediators, responses to capsaicin or heat dramatically increased to the extent that body temperature sufficed to activate nociceptors. Inflammatory mediators sensitize TRPV-1 function by various mechanisms; they may increase TRPV-1 expression levels in the membrane [1,5]. Shin et al. [7] suggested that TRPV-1 is a mediator of inflammatory hyperalgesia in DRG cells. They demonstrated that, by acting on a B2 bradykinin receptor, bradykinin stimulates production of 12-lipoxygenase metabolites, causing

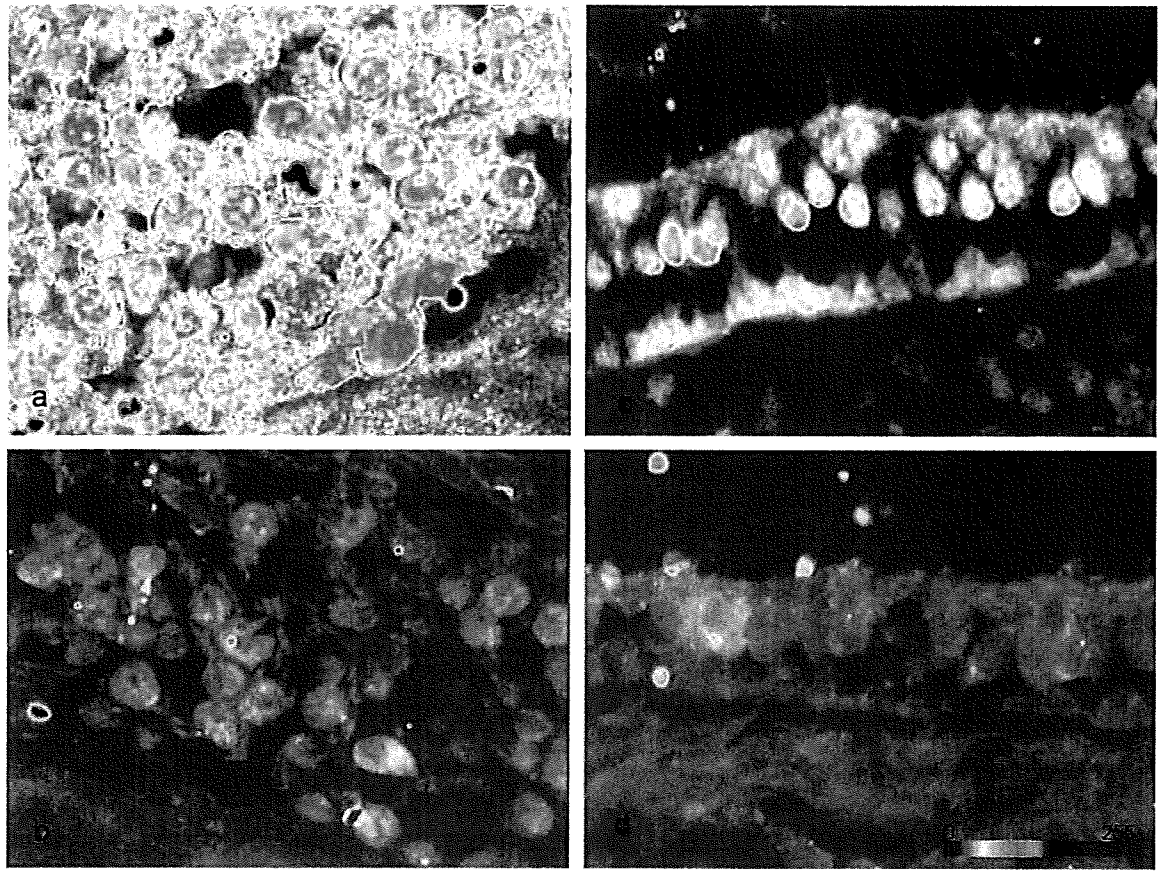

Figure 7. Pseudocolor images showing immunoreactivity to TRPV-3 and TRPV-4 in utricular macula. The spiral ganglion cells show immunoreactivity to TRPV-3 (a), which decreased following GM treatment. (b) The sensory cells show a marked fluorescent reaction to TRPV-4 (c), which decreased following GM treatment (d).

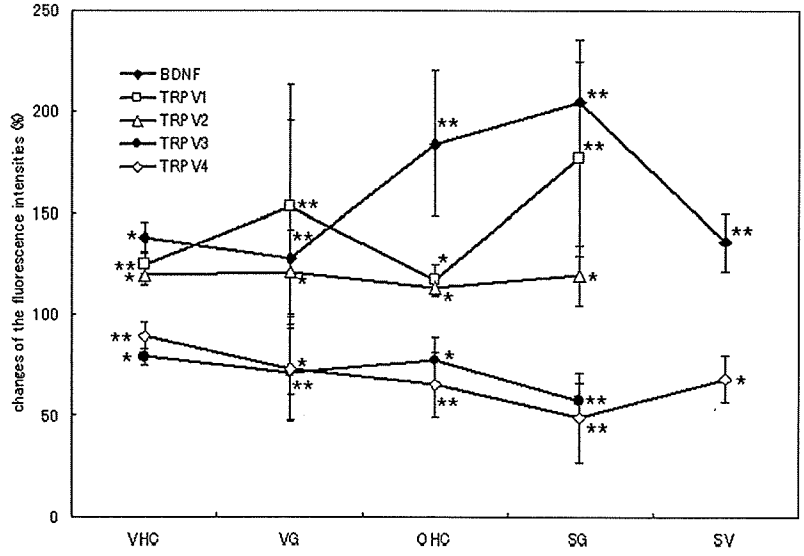

Figure 8. Changes in expression of BDNF and TRPVs in the GM-treated mouse inner ear. The fluorescent reaction to BDNF intensified in every part, i.e. vestibular sensory cells (VHC), vestibular ganglion cells (VG), cochlear hair cells (OHC), spiral ganglion cells (SG), and stria vascularis (SV). The fluorescent reaction to TRPV-1 and TRPV-2 also intensified, but weakened in TRPV-3 and TRPV-4 in general. $^{*}p<0.05$, $^{**}p<0.01$.

activation of TRPV-1 receptors. This TRPV-1 activation by bradykinin appeared to combine with other ligands and heat to increase intracellular calcium levels in DRG cells [8]. TRPV-1 up-regulation in the inner ear probably has discernible. An increase in expression would probably increase its depolarizing effects on sensory and ganglion cells, in turn increasing the basal firing rate and affecting stimulus-evoked discharges [2]. The clinical and pathological similarities between neuropathic pain and chronic tinnitus are well known [6]. As in the case of algesia caused by acute TRPV-1 stimulation, acute cochlear injury may produce tinnitus and acute vestibular insufficiency by activating the TRPV-1 receptor [2,6]. It is therefore suggested that TRPV-1 up-regulation may be a factor in the early emergence of tinnitus and vertigo during GM treatment [2,6].

Several observations link TRPV-1 activation or up-regulation to endogenous neuroprotective mechanisms in both the CNS and DRG. It was demonstrated recently that TRPV-1 activation contributes to the neuroprotective action of anandamide against ouabain-induced cell death in the striatum [9]. Furthermore, there is evidence that TRPV-1 expression is sensitive to growth factor levels. For example, exposure to brain-derived neurotrophic factor (BDNF) increases the capsaicin sensitivity of cultured vagal neurons [10]. Reports that TRPV-1 mRNA is up-regulated in DRG by glial cell line-derived neurotrophic factor (GDNF) and NGF treatment further suggest that this increase may be a growth factor-mediated neuroprotective mechanism in DRG cells. The present findings support a concomitant mechanism in the inner ear sensory cells and ganglia. BDNF is expressed constitutively by spiral ganglion cells and BDNF mRNA is transiently up-regulated in vestibular ganglion cells following unilateral instillation of GM into perilymph. Neurotrophins are also known to protect spiral and vestibular ganglion cell damage following GM treatment [11]. When BDNF is given simultaneously with GM, it minimizes the ototoxic effect of GM [12]. This study also revealed that the fluorescence of BDNF had increased in all parts of the inner ear after treatment with GM. Thus, TRPV-1 up-regulation seems to be one neuroprotective response of sensory and ganglion cells to the combined direct and indirect effects of GM intoxication. Since TRPV-1 is a non-specific cation channel, an up-regulation following GM treatment would be expected to facilitate depolarization of sensory cells as well as of spiral and vestibular ganglion cells. Depolarization per se has a potent neuroprotective effect in deafferented cultured spiral ganglion cells [13]. Spiral ganglion cell survival in vitro appears to reflect cumulative interactions in a depolarization-dependent autocrine (growth factor) pathway, an intracellular cyclic AMP-dependent pathway, and a calcium-calmodulin-dependent protein kinase pathway [14]. The latter two pathways are driven by depolarization-dependent calcium entry along L-type channels. These phenomena probably contribute to the neuroprotective effects of electrical stimulation and growth factors (BDNF, GDNF, and neurotrophic factor-3 (NT-3)) in preventing spiral ganglion degeneration following IHC destruction. Therefore, TRPV-1 up-regulation is a likely growth factor-mediated, positive feedback mechanism to enhance the depolarization-dependent calcium entry and growth factor release [2,14].

In the present study, up-regulation of TRPV-2 was also demonstrated in ganglion cells and the inner ear sensory cells. The functional significance of TRPV-2 in the latter is not yet completely clear. In TRPV-2-expressing cells, both hypotonic cell swelling and application of membrane stretch in patch clamp studies activated calcium influx, which could be blocked by ruthenium red or application of antisense oligonucleotides directed against TRPV-2. Because TRPV-2 is expressed in certain sensory neurons and in a wide range of stretch-sensitive cells, such as cardiac myocytes and vascular endothelial cells, the channel may be involved in stretch-activated calcium influx in these cells [1], indicating that TRPV-2 may participate in some part of hair cell motility and fluid homeostasis. Although regulatory mechanisms of TRPV-2 gating are poorly understood, reports suggest that growth factor (insulin-like growth factor I) and PI3-kinase signaling pathways enhance TRPV-2 activity [5]. The role of TRPV-2 in sensory neurons is not clear but a recent study on pain sensation reports an up-regulation of the TRPV-2 protein level in medium-sized DRG neurons after intraplantar injection of Freund's complete adjuvant, suggesting a role for TRPV-2 in peripheral sensitization during inflammation, possibly in the transduction of pain hypersensitivity to highly injurious temperature [15]. Probably because of its very high heat threshold as well as its differential distribution compared with TRPV-1, fewer studies have been carried out on pain focused on TRPV-2. Its predominant distribution in the neurotrophin-3-dependent subpopulation of DRG neurons [16], its increase in protein level following inflammation, its potential to heteromultimerize, and its propensity to be activated by 2-APB may be clues to its contribution to pain associated with inflammation or neuropathy [5]. As described above, the clinical and pathological similarity between neuropathic pain and chronic tinnitus is well known [6]. A similar mechanism should be considered in

the inner ear, suggesting that the up-regulation of TRPV-2 may also relate to the emergence of tinnitus and vertigo following GM treatment.

The present study revealed the down-regulation of TRPV-3 and TRPV-4 in the inner ear sensory and ganglion cells, and also a reduction of TRPV-4 in stria vascularis and vestibular dark cells. This contrasts with the up-regulation of TRPV-1 and TRPV-2. It has been already reported that there was also a modest (about 50%) down-regulation of TRPV-4 mRNA after KM treatment in the spiral and vestibular ganglion and kidney, but not in the TG [2]. Since TRPV-4 is the dominant member of the TRPV family in the kidney, and as aminoglycosides are both nephrotoxic and ototoxic, this parallel down-regulation of TRPV-4 mRNA in the kidney and inner ear ganglia seems to reflect direct (possibly cytotoxic) effects of KM that are shared by the ganglia and kidney [2].

Concerning the functional significance of the down-regulation of TRPV-3 and TRPV-4 in the inner ear following GM treatment, a neuroprotective function could be proposed. Several studies support this hypothesis. TRPV-4 knockout mice aged 8 weeks exhibited normal auditory brainstem response thresholds, but those aged 24 weeks had significantly higher thresholds. The auditory threshold shift was significantly greater in TRPV4 knockout mice than in TRPV4+/+ mice at 1 week after the acoustic overstimulation with 128 dBSPL. The TRPV-4 channel is not related to ultimate cell death but is necessary for maintaining cochlear function under stressful conditions, such as aging and acoustic injury [17]. These results indicate that the TRPV-4 participates in neuroprotection of the inner ear.

TRPV-3 and TRPV-4, both expressed in the CNS, are sensitive to temperature changes within the physiological range (TRPV3 around 37°C, TRPV-4 about 25–43°C). These two channels play a role in regulating intracellular Ca homeostasis, as we recently demonstrated in nigral dopaminergic neurons. TRPV-3/TRPV-4 may also potentiate the neuroprotective effects of hypothermia on CA1 neurons. To further test the hypothesis that sealing of TRPV-3 and/or TRPV-4 channels by lowering temperature contributes (in addition to other factors) to the neuroprotective effect of hypothermia on brain tissue, it will be necessary to establish that metabolic and oxidative stress does indeed activate these channels. Activation of TRPV-4 channels is likely, as these channels are sensitive to cell swelling and low pH, which are always associated with brain ischemia. Although a link between ischemia and TRPV-3 channel activation has not yet been reported, this possibility is consistent with the recently suggested notion that all members of the TRP channel superfamily can respond to oxidative stress [18]. The known neuroprotective effect of hypothermia may be at least partly due to sealing of temperature-sensitive TRP channels (TRPV-3 and/or TRPV-4).

It has also been suggested that hypo-osmotic stimulation can induce NO production by an increase in Ca^{2+}, which is presumably mediated by activation of TRPV-4 in the OHCs [19]. Following GM treatment, an inducible type of NOS has been expressed in the inner ear tissue, which generates the large amount of NO resulting in a degeneration of sensory cells and ganglia [4]. The down-regulation of both TRPV-3 and TRPV-4 may block entry of Ca^{2+} into the sensory and ganglion cells, which may give neuroprotection.

The present study also revealed a down-regulation of TRPV-4 in stria vascularis as well as in vestibular dark cells. TRPV-4, localized mainly in the inner ear fluid transporting cells, seems to have an important function in inner ear fluid homeostasis. GM treatment induces a slowly progressive degeneration of stria vascularis and vestibular dark cells [20]. It needs to be clarified if the down-regulation of TRPV-4 in secretory epithelia is due to a direct effect of GM or due to degeneration of these tissues. Whichever the reason, down-regulation of TRPV-4 in these epithelia must induce a disturbance of inner ear fluid homeostasis and may also be related to a decrease in endolymph production.

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ORIGINAL ARTICLE

Effect of inner ear blood flow changes in Ménière's model mice

MASAYA TAKUMIDA[1], NANA AKAGI[2] & MATTI ANNIKO[3]

[1]*Department of Otolaryngology, Hiroshima University Faculty of Medicine, Hiroshima,* [2]*International Medical Center of Japan, Tokyo, Japan and* [3]*Department of Otolaryngology, Head and Neck Surgery, University Hospital, Uppsala, Sweden*

Abstract

Conclusions. The endolymphatic sac (ES) is important for inner ear fluid homeostasis. A dysfunctional ES can cause vertigo attacks following additional stress such as a sudden change in endolymphatic volume and/or pressure, or restricted inner ear blood flow. *Objective.* The purpose of this study was to elucidate the mechanism of vertigo attacks in Ménière's disease. *Materials and methods.* Adult CBA/J mice were given an intratympanic injection of lipopolysaccharide and an intraperitoneal injection of aldosterone. These 'model' animals had epinephrine or sodium nitroprusside (SNP) instilled into the middle ear cavity. Cochleae, vestibules, and endolymphatic sacs were studied morphologically by light microscopy. *Results.* The injection of epinephrine into the model animals reduced the endolymphatic hydrops in the cochlea, but also produced mild hydrops in the vestibule, which was never observed in untreated (control) animals. The ES did not react to epinephrine in the normal way. Injection of SNP did not cause any changes.

Keywords: *Endolymphatic sac, inner ear blood flow, morphology, epinephrine, sodium nitroprusside, Ménière's disease*

Introduction

Ménière's disease (MD) is a disorder of the inner ear characterized by intermittent episodes of vertigo, fluctuating sensorineural hearing loss, tinnitus, and aural pressure. Currently, there is no universally accepted explanation for the underlying pathophysiology of this disease, although the histopathological findings in MD have been described in extensive studies on temporal bones. It is assumed that endolymphatic hydrops (EH) is the pathological feature most descriptive of MD. EH is the most common pathological finding in patients and can be induced experimentally in animals, but the disease with its clinical manifestations is not reproducible in the laboratory and can only be studied in patients [1].

A number of methods have been developed to simulate an animal model of MD. Since its introduction by Kimura and Schuknecht in 1965 [2], surgical induction (by obliteration of the endolymphatic duct and sac) of EH in the guinea pig has become the standard model for the study of MD. This procedure has been readily adopted by some investigators because it reliably produces both histological EH and hearing loss. However, this animal model does not produce anything resembling attacks of vertigo, even though a predictable low-tone hearing loss can ensue [3,4]. In this standard model, produced by surgical obliteration, EH is induced by destroying the endolymphatic sac (ES) and obliterating the endolymphatic duct (ED) with bone wax [2]. Recently, Dunnebier et al. [5] induced mild EH by total dissection of the extra-osseous part of the ES adjacent to the sigmoid sinus to obstruct the venous outflow to the sinus and produce mild fibrosis of the most distal portion of the sac. Although several modifications of the standard animal model, including Dunnebier's model [5], have been developed, which produced varying degrees of EH, they were still too destructive or could not be standardized sufficiently to be superior to the standard model [6].

In contrast to ablation, 'over-production' models have been developed that, like the surgical model, elicit both histological EH and hearing loss [3,4,6]. These models include the introduction of cholera toxin into the inner ear [7], long-term administration

Correspondence: Masaya Takumida MD, Department of Otolaryngology, Hiroshima University Faculty of Medicine, 1-2-3 Kasumicho, Minamiku, Hiroshima 734-8551, Japan. E-mail: masati@hiroshima-u.ac.jp

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