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ORIGINAL PAPER

Oxidative Stress, Redox Homeostasis and Cellular Stress Response in Ménière's Disease: Role of Vitagenes

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Abstract Ménière's disease (MD) is characterized by the triad of fluctuating hearing loss, episodic vertigo and tinnitus, and by endolymphatic hydrops found on post-mortem examination. Increasing evidence suggests that oxidative stress is involved in the development of endolymphatic hydrops and that cellular damage and apoptotic cell death might contribute to the sensorineural hearing loss found in later stages of MD. While excess reactive oxygen species (ROS) are toxic, regulated ROS, however, play an important role in cellular signaling. The ability of a cell to counteract stressful conditions, known as cellular stress response, requires the activation of pro-survival pathways and the production of molecules with anti-oxidant, anti-apoptotic or pro-apoptotic activities. Among the cellular pathways conferring protection against oxidative stress, a key role is played by vitagenes, which include heat shock proteins (Hsps) as well as the thioredoxin/thioredoxin reductase system. In this study we tested the hypothesis that in MD patients measurable increases in markers of cellular stress response and oxidative stress in peripheral blood are present. This study also explores the hypothesis that changes in the redox status of glutathione, the major endogenous antioxidant, associated with abnormal expression and activity of carbonic anhydrase can contribute to increase oxidative stress and to disruption of systemic redox homeostasis which can be associated to possible alterations on vulnerable neurons such as spiral ganglion neurons and consequent cellular degeneration. We therefore evaluated systemic oxidative stress and cellular stress response in patients suffering from Meniere's disease (MD) and in age-matched healthy subjects. Systemic oxidative stress was estimated by measuring protein oxidation, such as protein carbonyls (PC) and 4-hydroxy-nonenal (HNE) in lymphocytes of MD patients, as well as ultraweak luminescence (UCL) as end-stable products of lipid oxidation in MD plasma and lymphocytes, as compared to age-matched controls, whereas heat shock proteins Hsp70 and thioredoxin (Trx) expression were measured in lymphocytes to evaluate the systemic cellular stress response. Increased levels of PC ($P < 0.01$) and HNE ($P < 0.05$) have been found in lymphocytes from MD patients with respect to control group. This was paralleled by a significant induction of Hsp70, and a decreased expression of Trx ($P < 0.01$), whereas a significant decrease in both plasma and lymphocyte ratio reduced glutathione GSH) vs. oxidized glutathione (GSSG) ($P < 0.05$) were also observed. In conclusion, patients affected by MD are under condition of systemic oxidative stress and the induction of vitagenes Hsp70 is a maintained

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response in counteracting the intracellular pro-oxidant status generated by decreased content of GSH as well as expression of Trx. The search for novel and more potent inducers of vitagenes will facilitate the development of pharmacological strategies to increase the intrinsic capacity of vulnerable ganglion cells to maximize antidegenerative mechanisms, such as stress response and thus cytoprotection.

Keywords Glutathione · Redox homeostasis · Oxidative stress · Ménière's disease · Neurodegenerative diseases

Introduction

Ménière's disease (MD) is characterized by the triad of fluctuating hearing loss, episodic vertigo and tinnitus, and by endolymphatic hydrops found on post-mortem examination [1, 2]. The cause of Meniere's disease remains unclear, although numerous causative factors have been considered in the development of hydrops and in the pathogenesis of related cochleovestibular dysfunction [3]. Experimental data both from human as well as animal models of the disorder have generally failed to determine the mechanism by which ELH or related pathology causes hearing loss. However, a limited number of detailed ultrastructural studies have demonstrated significant reductions in dendritic innervation densities, raising the possibility that neurotoxicity plays an important role in the pathology of MD [3]. Increasing evidence suggests that oxidative stress is involved in the development of endolymphatic hydrops and that cellular damage and apoptotic cell death might contribute to the sensorineural hearing loss found in later stages of MD [4]. Furthermore, it is well known that reduced expression and/or activity of antioxidant proteins lead to oxidative stress, accelerated aging and neurodegeneration [5–8].

While excess reactive oxygen species (ROS) are toxic, regulated ROS, however, play an important role in cellular signalling [9]. The ability of a cell to counteract stressful conditions, known as cellular stress response, requires the activation of pro-survival pathways and the production of molecules with anti-oxidant, anti-apoptotic or pro-apoptotic activities [6]. Among the cellular pathways conferring protection against oxidative stress, a key role is played by vitagenes, which include heat shock proteins (Hsps) heme oxygenase-1 and Hsp70, as well as the thioredoxin/thioredoxin reductase system [7–10]. Heat shock response contributes to establish a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders. Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing stress responses [6–8]. When appropriately activated, cellular stress response has the capability to restore cellular homeostasis and rebalance redox equilibrium. Activation of antioxidant pathways is particularly important for neural cells with relatively weak endogenous antioxidant defenses, such as spiral ganglion neurons which are a centrally involved in the pathogenesis of MD. Perturbation of redox status, overloading of the product of polyunsaturated fatty acid peroxidation (hydroxynonenals, HNE) or protein carbonyls (PC) can disrupt redox homeostasis. Moreover it is known that normal auditory function depends on maintenance of the unique ion composition in the endolymph. Hence, carbonic anhydrase in the inner ear has been suggested to play an important role in maintaining the ion concentration and regulating fluids of the inner ear [11–13].

In this study we tested the hypothesis that neurotoxicity is an important primary mediator of injury in Ménière's disease and that in MD patients measurable increases in markers of cellular stress response and oxidative stress in peripheral blood are present. This study also explores the hypothesis that changes in the redox status of glutathione, the major endogenous antioxidant, associated with abnormal expression and activity of carbonic anhydrase can contribute to increase oxidative stress and to disruption of systemic redox homeostasis which can be associated to possible alterations on vulnerable neurons such as spiral ganglion neurons and consequent cellular degeneration.

Materials and Methods

Chemicals

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3-tetraethoxypropane, purified bovine blood SOD, NADH, glutathione (GSH), glutathione disulfide (GSSG), β-NADPH (type 1, tetrasodium salt), and glutathione reductase (GR; Type II from Bakers Yeast), were from Sigma Chemicals Co, St. Louis (USA). All other chemicals were from Merck (Germany) and of the highest grade available.

Ethical Permission

The study was approved by the local Ethics Committee and informed consent was obtained from all patients.

Patients

We enrolled 39 patients (20 males and 19 females, with an average age of 49.5 years) with Ménière's disease according to the diagnostic scale of the Committee on Hearing and Equilibrium of the American Academy of

Otolaryngology-Head and Neck Surgery published in 1995 for MD [14] (two or more definitive spontaneous episodes of vertigo 20 min or longer, audiometrically documented hearing loss on at least one occasion, tinnitus or aural fullness in the treated ear), and 34 age-matched healthy subjects. This control group, including 15 males and 19 females, with an average age of 39.5 years, had no cochleo-vestibular disorders and no systemic diseases. Criteria of exclusion were determined by audiometric and clinical examinations, for those patients showing tympanic membrane perforation, otosclerosis, infectious or neoplastic diseases of the ear and of the acoustic nerve. All patients were administered the Profile of Mood States (POMS), which evaluates emotions and stress of past weeks: Tension-Anxiety (T-A), Depression-Dejection (D), Anger-Hostility (A-H), Vigor-Activity (V), Fatigue (F), Confusion-Bewuilderment (C). The Profile of Mood States original scale contains 65 self-report items using the 5-point Likert Scale. Participants can choose from 0 (not at all) to 4 (extremely) [15, 16]. In addition all subjects were given a tinnitus questionnaire consisting of 40 multiple choice questions to define the impact of symptoms on the patient life. In the group of patients with diagnosis of Ménière's disease, the grade of severity for each patient (Table 1) was established on the basis of the vertigo attack frequency over a year (from 2 to 8 crisis), the intensity and the duration of symptoms (from a few days, to some weeks, to a month in the most severe case) [17, 18]. In this study group, the hearing loss degree was assessed instrumentally allowing staging of the disease in MD patients, as reported in Table 2.

Sampling

Blood (6 ml) was collected after an overnight fast by venopuncture from an antecubital vein into tubes containing 500 mM EDTA as anticoagulant. Immediately after sampling, two blood aliquots were separated: first 2 ml were centrifuged at 10.000 × g for 1 min at 4°C to separate plasma from red blood cells; the remaining aliquot (4 ml) was utilized for lymphocytes purific. All samples were stored at −80°C until analysis.

Table 1 Crisis frequency in a year

Crisis frequency		Crisis duration	
2 crisis per year	3 patient (7.6%)	Less than 1 h	8 (20.4%)
4 crisis per year	19 patients (48.71%)	From 1 to 7 h	18 (46.1%)
5 crisis per year	8 patients (20.4%)	From 8 to 21 h	10 (25.6%)
7 crisis per year	6 patients (15,3%)	More than 30 h	3 (7.7%)
8 crisis per year	3 patient (7,6%)		

Table 2 Staging of patients with MD at admission

Stage of MD	Tone average (dB)	Numbers of patients	Percentage
Mild hearing threshold	20–40	7	17.9
Milder hearing threshold	40–70	21	53.8
Severe hearing threshold	70–90	11	28.2

Lymphocytes Purification

Lymphocytes from peripheral blood were purified by using the Ficoll Paque System following the procedure as suggested by the manufacturer (GE Healthcare, Piscataway, NJ).

Western Blot Analysis

The lymphocyte pellet was homogenized and centrifuged at 10,000 × g for 10 min and the supernatant was used for analysis after dosage of proteins. Aliquot (40 μg) of protein extract was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) by using the slab gel system of Pharmacia (GE Healthcare, Piscataway, NJ), transferred overnight to nitrocellulose membrane and the non specific binding of antibodies was blocked with 3% non-fat dry milk in PBS. Membrane were then probed with a monoclonal mouse anti-Hsp72 antiboby (SPA-810, Stressgen) that recognizes only the inducible form. Instead, carbonic anhydrase II (CAII), DPNH, HNE, and Trx were performed using polyclonal rabbit antibodies: (ab6621, Abcam, Cambridge, UK), (SPA-895, Stressgen), (V0401, DAKO), (HNE11-S Alpha Diagnostic International), (07-613, Upstate Biotechnology), respectively. Then, a goat polyclonal antibody specific for β-actin was used for loading control (1:1,000). For detection, blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) when probing CAII, DPNH, HNE and Trx, whereas a horseradish peroxidase-conjugated goat anti-mouse (IgG) was used in the case of Hsp70 and horseradish peroxidase-conjugated anti-goat (IgG) for detection of β-actin followed by ECL chemiluminescence (Amersham).

Immunoreactive bands were quantified by scanning Western blot imaged films with a laser densitometer (LKB-Ultrascan, XL model). Several blots were prepared with all samples and each sample was immunoblotted 3–4 times to check variability in the immunodetection signal. Molecular weights of the proteins detected were determined by using a standard curve obtained with proteins of known molecular weight.

Glutathione and Glutathione Disulfide Assay

GSH and GSSG were measured by the NADPH-dependent GSSG reductase method as previously reported [19, 20]. Lymphocytes were homogenized on ice for 10 s in 100 mM potassium phosphate, pH 7.5, which contained 12 mM disodium EDTA. For total glutathione, an aliquots (0.1 ml) of homogenates were immediately added to 0.1 ml of a cold solution containing 10 mM DTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The samples were then mixed by tilting and centrifuged at 12,000 g for 2 min at 4°C. An aliquot (50 µl) of the supernatant was added to a cuvette containing 0.5 U of GSSG reductase in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml. The formation of a GSH-DTNB conjugate was then measured at 412 nm. The reference cuvette contained equal concentrations of DTNB, NADPH and enzyme, but not sample. For assay of GSSG, aliquots (0.5 ml) of homogenate were immediately added to 0.5 ml of a solution containing 10 mM N-ethylmaleimide (NEM) and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample was mixed by tilting and centrifuged at 12,000 g for 2 min at 4°C. An aliquot (500 µl) of the supernatant was passed at one drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA) that had been washed with methanol followed by water. The column was then washed with 1 ml of buffer 1. Aliquots (865 µl) of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. GSH and GSSG standards in the ranges between 0–10 nmol and 0.010–10 nmol, respectively, added to control samples were used to obtain the relative standard curves, and the results were expressed in nmol of GSH or GSSG, respectively, per ml or mg protein.

Spontaneous Ultraweak Chemiluminescence Assay

Measurement of chemiluminescence in blood samples was accomplished according to the method of Flecha [20]. Briefly, aliquots (0.5 ml) of plasma were diluted 1:1 with 30 mM phosphate buffer (pH 7.4), whereas lymphocyte pellet was homogenized and centrifuged at 10,000 × g for 10 min. Before aliquots (0.5 ml) of the supernatant were taken and diluted 1:1 with 30 mM phosphate buffer (pH 7.4) at 0–4°C and centrifuged at 10,000 g for 3 min at 0–4°C. Then spontaneous ultraweak chemiluminescence (UCL) was measured in the supernatant at 30°C with a Turner TD 20/20 luminometer. The sensitivity was adjusted to 50%, and results were expressed as luminescence units/mg protein.

Determination of Protein

Proteins were estimated by the BCA protein assay method [21] using bicinchoninic acid (BCA) reagent.

Statistical Analysis

Results were expressed as means ± SEM of n=39 experiments, each of which were performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $P < 0.05$.

Results

As a consequence of oxidative stress in tissues and organs, protein and lipid oxidation occur [22]. In this study, protein oxidation has been evaluated by measuring the amount of protein carbonyls (PC) by Western Blot analysis. As shown in Fig. 1, DPNH-reactive PC levels resulted significantly higher ($P < 0.01$) in lymphocytes of MD patients than in control subjects .

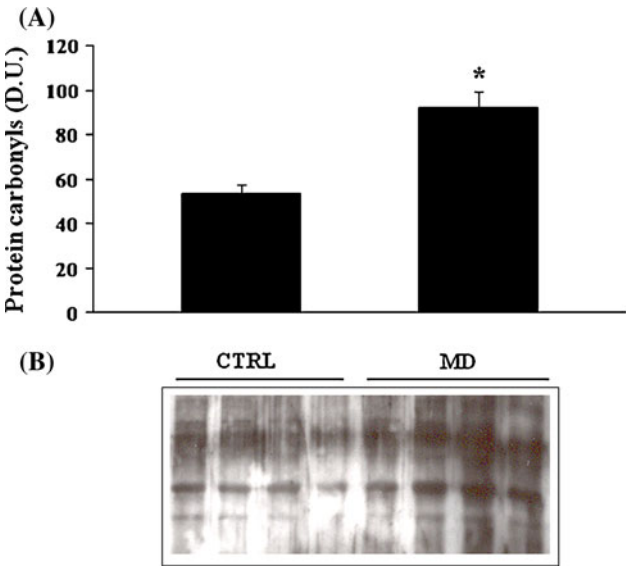

Fig. 1 Protein Carbonyls (DPNH-derivative, PC) in lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. Protein carbonyls were measured as described in methods. The *bar graph* shows the densitometric evaluation and values are expressed as mean ± standard error of mean of 3 independent analyses on 34–39 subjects per group. A representative immunoblot is shown. *β*-actin has been used as loading control. *CTRL* control, *MD* Ménière disease patients. (*) $P < 0.01$ vs. control

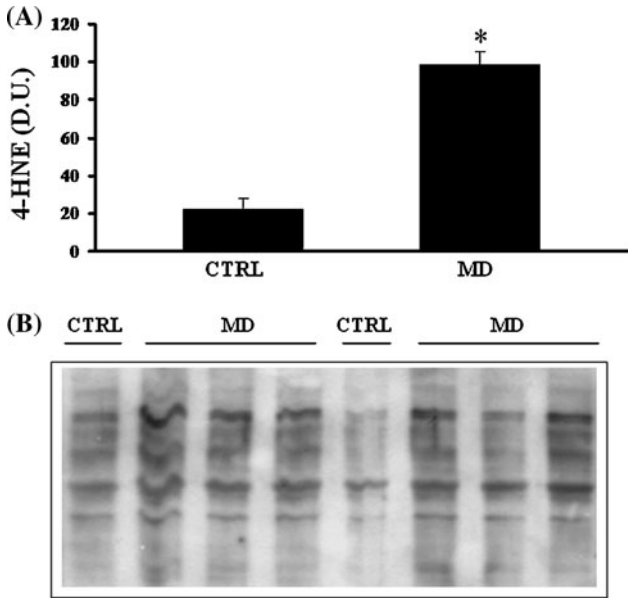

Fig. 2 4-hydroxynonenals (HNE) in lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. HNE were measured as described in methods. The *bar graph* shows the densitometric evaluation and values are expressed as mean ± standard error of mean of 3 independent analyses on 34–39 subjects per group. A representative immunoblot is shown. *β*-actin has been used as loading control. *CTRL* control, *MD* Ménière disease patients. * $P < 0.01$ vs. control

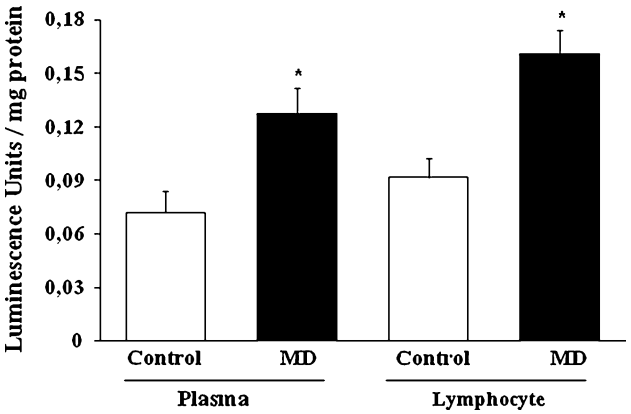

Fig. 3 Spontaneous ultraweak chemiluminescence (UCL) in plasma and lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. UCL was measured as described in methods. *CTRL* control, *MD* Ménière disease patients. * $p < 0.01$ vs. control

Following free radical attack, 4-hydroxynonenal (HNE) are formed from arachidonic acid or other unsaturated fatty acids [22] and bind, by Michael addition, to proteins particularly at cysteine, hystidine, or lysine residues [23]. Under conditions of oxidative stress, lipid oxidation, measured by HNE, can also occur in different tissues, including the brain and peripheral blood stream [24]. Examination of HNE levels in lymphocyte samples (Fig. 2) has shown a significant elevation ($P < 0.05$) of protein-bound HNE in MD patients respect to control subjects. Other marker of oxidative stress is ultreweak luminescence, which has been proposed as index of lipid peroxidation in diabetic patients [7, 25]. We demonstrate that plasma and lymphocytes levels of ultraweak luminescence (Fig. 3) were significantly increased in MD patients ($P < 0.01$) with respect to controls.

Consistent with others, who showed that oxidative stress and altered thiol status in degenerating brain diseases correlates with systemic redox imbalance and oxidative stress, as in AD. Consequently, the content of total GSH, reduced and oxidized glutathione and the GSH/GSSG ratio in the plasma and in lymphocytes of MD patients was determined as a measure of the antioxidant status and compared with the levels of control group (Table 3) [26–32]. We report that plasma and peripheral lymphocytes from MD patients showed a significantly decreased GSH levels ($P < 0.05$) and corresponding significantly increased GSSG levels ($P < 0.05$) (Table 3). These changes significantly decreased the GSH/GSSG *ratio* in MD plasma and lymphocytes compared to controls (Table 3).

Our lab previously demonstrated upregulation of protective proteins in cells exposed to oxidative stress [11–32]. Consistent with these prior findings, in the present study we observed an increased expression of cytoprotective proteins Hsp72 and a decreased expression in TRX ($P < 0.01$) (Figs. 4, 5) in lymphocytes of MD patients compared to controls [33–36]. Changes in expression of these two proteins seemed to be consequent to a strong oxidant environment underlying the pathogenesis of this neurodegenerative disorder. Moreover, it is known that normal auditory function depends on maintenance of the unique ion composition in the endolymph. Hence, carbonic anhydrase in the inner ear has been suggested to play an important role in maintaining the ion concentration and regulating fluids of the inner ear. Consistent with this notion, we report that in MD patients a significant ($P < 0.05$) increase of carbonic anhydrase type II expression occur in comparison with controls levels (Fig. 6).

Discussion

Classically, the tetrad of symptoms–episodic vertigo, fluctuating sensorineural hearing loss, tinnitus, and ear blockage–associated with the histopathological correlate endolymphatic hydrops has been diagnosed as Ménière's disease [37–41]. The cause of Meniere's disease remains unclear, although numerous causative factors have been considered in the pathogenesis of related cochleovestibular dysfunctions. Specifically, key etiological agents that have been identified as playing a role in the clinical course of

Table 3 Plasma and lymphocyte content of total, reduced (GSH) and oxidized (GSSG) glutathione in control and MD patients

	Plasma (nmol/ml)		Lymphocyte (nmol/mg protein)	
	Control	MD	Control	MD
Total GSH	15.9 ± 2.0	9.22 ± 3.1*	8.86 ± 0.5	5.5 ± 0.6*
GSH	14.33 ± 2.1	9.09 ± 1.6*	7.82 ± 0.6	4.48 ± 0.5*
GSSG	0.149 ± 0.01	0.155 ± 0.02	0.081 ± 0.01	0.105 ± 0.01*
Ratio GSH/GSSG	96.1 ± 12	58.6 ± 14*	96.5 ± 10	42.6 ± 7.9*

* Significantly different from control ($P < 0.05$)

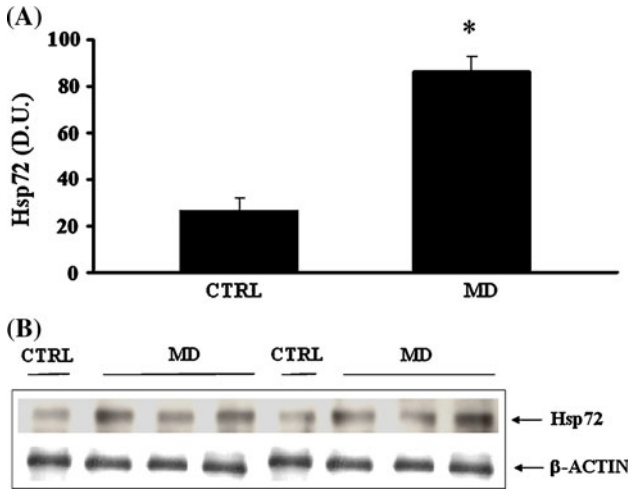

Fig. 4 Hsp72 expression in lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. Hsp72 were measured as described in methods. The *bar graph* shows the densitometric evaluation and values are expressed as mean ± standard error of mean of 3 independent analyses on 34–39 subjects per group. A representative immunoblot is shown. β-actin has been used as loading control. *CTRL* control, *MD* Ménière disease patients. * $P < 0.01$ vs. control

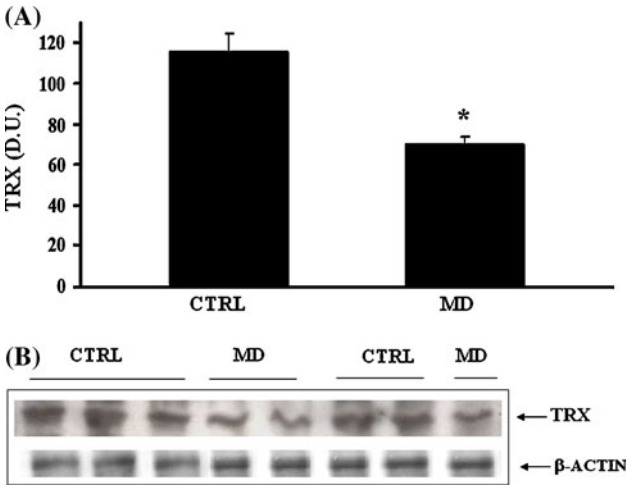

Fig. 5 Thioredoxin (TRX) protein expression in lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. TRX was measured as described in methods. The *bar graph* shows the densitometric evaluation and values are expressed as mean ± standard error of mean of 3 independent analyses on 34–39 subjects per group. A representative immunoblot is shown. β-actin has been used as loading control. *CTRL* control, *MD* Ménière disease patients. * $P < 0.01$ vs. control

tinnitus (e.g., noise exposure, stress) may serve as "triggers" or stressors (or both), resulting in interference in normal biochemical and physiological function of sensorineural structures in the inner ear or in neural structures in the brain.

Endolymphatic hydrops is an important histopathological hallmark of Ménière's disease. Experimental data from human temporal bones as well as animal models of the disorder have generally failed to determine the mechanism by which endolymphatic hydrops or related pathology causes hearing loss. Hair cell and spiral ganglion cell counts in both human and animal case studies have not, for the most part, shown severe enough deterioration to explain associated severe sensorineural hearing loss. However, a limited number of detailed ultrastructural studies have demonstrated significant reductions in dendritic innervation densities, raising the possibility that neurotoxicity plays an important role in the pathology of Ménière's disease as well as experimental endolymphatic hydrops. The association of Meniere's syndrome with endolymphatic hydrops has led to the formation of a central hypothesis: many possible etiologic factors lead to hydrops, and hydrops in turn generates the symptoms. However, this hypothesis of hydrops as being the final common pathway has not been proven conclusively. The major pathophysiological alterations underlying the tetrad of inner-ear symptomatology, otherwise diagnosed as Ménière's disease, has also been associated clinically with perfusion asymmetries in brain, identified by nuclear medicine brain imaging (single-photon emission computed tomography [SPECT] of brain), and reflects an interference in homeostasis in the blood–brain labyrinth or blood–brain barriers, with a resulting secondary endolymphatic hydrops.

Increasing evidence suggests that oxidative stress is involved in the development of endolymphatic hydrops and that cellular damage and apoptotic cell death might contribute to the sensorineural hearing loss found in later stages of MD [42]. Oxidation–reduction (redox) based

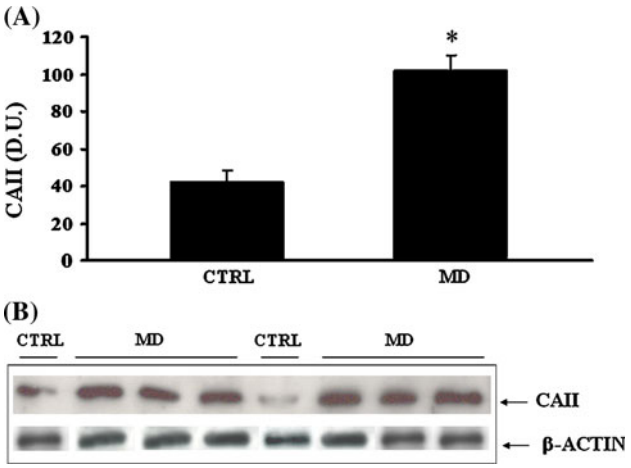

Fig. 6 Carbonic Anhydrase II (CA) expression in lymphocytes of control healthy volunteers and Ménière Diseased (MD) patients. CAII was measured as described in methods. The *bar graph* shows the densitometric evaluation and values are expressed as mean ± standard error of mean of 3 independent analyses on 34–39 subjects per group. A representative immunoblot is shown. *β*-actin has been used as loading control. *CTRL* control, *MD* Ménière disease patients. * $P < 0.01$ vs. control

regulation of gene expression appears to be a fundamental regulatory mechanism in cell biology. This basic information has been exploited to develop novel strategies in clinical therapeutics.

All living cells are involved in reduction–oxidation (redox) activities that are essential to cellular function. Many such processes, such as mitochondrial respiration, monooxygenase, and oxidase activities, chemically reduce molecular oxygen to form reactive oxygen. A resulting redox imbalance in favor of oxidation (oxidative stress) may result in a cellular oxidative stress response [11, 43]. The chemical reductive processes for the formation of reactive oxygen species (mostly superoxide, hydrogen peroxide, hydroxyl radical) are supported primarily by reduced pyridine nucleotides, such as NADH and NADPH. Thus, higher rates of reactive oxygen production may occur when the redox state of the cell becomes more negative, a process termed reductive stress that may originate in the cytosol or in the mitochondrial compartment [8, 11]. To minimize adverse cellular effects resulting from constitutive or excessive exposure to reactive oxygen, most cells have an elaborate defense system that includes a broad spectrum of chemical and enzyme scavengers for the oxidizing radicals and electrophiles. GSH is an integral oxidant scavenger, reacting as either a one-electron donor to radicals or a two-electron donor to electrophiles, and occurs in all mammalian cell types [28]. Protein thiols play a key role in redox sensing, and regulation of cellular redox state is a crucial mediator of multiple metabolic, signalling and transcriptional processes [44]. Under optimal conditions long-term health protection is accomplished by protein homeostasis, a highly complex network of molecular interactions that balances protein biosynthesis, folding, translocation, assembly/disassembly, and clearance. Protein quality control is a critical feature of intracellular homeostasis [45]. When conformationally challenged aggregation-prone proteins are expressed, the resulting unfolded or misfolded proteins are rapidly degraded via the ubiquitin–proteasome pathway. The ability of a cell to counteract stressful conditions, also known as cellular stress response, requires the activation of pro-survival pathways as well as production of molecules endowed with anti-oxidant and anti-apoptotic activities, which is under control of protective genes called vitagenes [8]. Generally, molecular chaperones help hundreds of signaling molecules to keep their activation-competent state, and regulate various signaling processes ranging from signaling at the plasma membrane to transcription. Besides these specific regulatory roles, recent studies have revealed that chaperones act as genetic buffers stabilizing the phenotypes of various cells and organisms [11, 26]. This may be related to their low affinity for the proteins they interact with, which means that they represent weak links in protein networks [11, 26]. Chaperones may uncouple protein, membrane, organelle and transcriptional networks during stress, which gives the cell additional protection. The same networks are preferentially remodeled in various diseases and aging, which may help us to design novel therapeutic and anti-aging strategies [11, 43]. Among the cellular pathways involved in the so called "programmed cell life" conferring protection against oxidative stress, a key role is played by the products of vitagenes [11, 43]. These include members of the heat shock protein (Hsp) family, such as Hsp72 and the thioredoxin/thioredoxin reductase antioxidant system [11, 43].

In this study we tested the hypothesis that neurotoxicity is an important primary mediator of injury in Ménière's disease and may be reflected in measurable increases in markers of cellular stress response and oxidative stress in the peripheral blood of patients with Meniere's disease. The results of our study are in agreement with the first report showing that free-radical generation might contribute to the ototoxicities of several chemical agents, as demonstrated utilizing electron paramagnetic resonance (EPR) spectrometry to detect directly ototoxicant- induced reactive oxygen species formation in cochlear tissue. In this interesting study [46], guinea pig cochlear explants exoposed to various ototoxicants, such as gentamicin, kanamycin, ethacrynic acid, furosemide, cisplatin, trimethyltin chloride or quinine and EPR spectra measured after addition of 5,5-dimethylpyrroline-N-oxide (DMPO) as of spin trap. The spectra obtained were consistent with the the proposal that various ototoxic agents are able to induce

oxidative stress thus providing a plausible mechanism by which these ototoxic insults target cochlear tissues and cause auditory dysfunction [46].

Our finding of increased expression of Hsp70, associated with significant changes in the systemic redox status as the reduction in i) the content of GSH; ii) the ratio of GSH/GSSG ratio and iii) the expression of Trx indicate, are relevant to the involvement of oxidative stress in the pathogenesis of Ménière disease. Although the scientific evidence for the cause of MD has not been sufficiently established, it is well known that MD patients with severe symptoms often have some mental stress [30]. Stress-related hormones have been measured to assess the presence of stress. However, it is difficult to evaluate the stress responses with MD patients by stress-related hormones, because various factors affect the hormonal reaction. In general, the stress response appears to increase the expression of heat-shock proteins in cells [47]. HSPs act to protect cells against various kinds of stress such as oxidative free radicals and toxic metal ions [30]. Hsp70 proteins function as molecular chaperones and are involved in cell survival under stress condition [11, 43]. Consistent with this notion, polymorphic analysis of the heat-shock protein 70 gene (HSPA1A) in Ménière's disease revealed that a single nucleotide polymorphism (SNP) 190 G/C in HSP70-1 (HSPA1A) was found to be a factor associated with Ménière's disease (MD) [30]. It is suggested that SNP 190 G/C could be scientific evidence to prove the relation between MD and stress as a trigger factor. In fact The HSP family, especially Hsp70, acts to defend against a number of cellular stresses, while it also acts as molecular chaperones [11]. If the *HSP70* gene had polymorphisms, alteration of either qualitative or quantitative HSP70 expression could change differential sensitivity to stresses. Therefore, *HSP70* polymorphisms could be generally associated with susceptibility to diseases [30]. Polymorphisms in the *HSP70* gene have been studied with regard to their associations with various conditions related to oxidative stress, such as aging and inflammation. On the other hand, it has been suggested that HSPs act in autoimmunity. Therefore, polymorphisms of the *HSP70* gene have been investigated in autoimmune diseases including systemic lupus erythematosus (SLE), Graves' disease, sarcoidosis, insulin-dependent diabetes mellitus (IDDM), and asthma [48]. In this study we tested the hypothesis that neurotoxicity is an important primary mediator of injury in Ménière's disease and may be reflected in measurable increases in markers of cellular stress response and oxidative stress in the peripheral blood of patients with Meniere's disease. This study also validate the hypothesis that changes in the redox status of glutathione, the major endogenous antioxidant, associated with abnormal expression and activity of the antioxidant protein thioredoxin, as well as carbonic anhydrase can contribute to increase oxidative stress with disruption of redox homeostasis in vulnerable neurons such as spiral ganglion neurons and consequent cellular degeneration.

It is known that normal auditory function depends on maintenance of the unique ion composition in the endolymph. Hence, carbonic anhydrase in the inner ear has been suggested to play an important role in maintaining the ion concentration and regulating fluids of the inner ear. Our finding of an increased expression of carbonic anhydrase in MD patients may be relevant to the pathogenesis of the disease in view of the finding showing that bicarbonate a product of carbonic anhydrase enhances peroxidase activity of Cu,Zn-superoxide dismutase, an alternative function that has been implicated in the onset and progression of familial amyotrophic lateral sclerosis. Carbonic anhydrase is a sufhydryl protein particularly vulnerable to oxidative damage, as demonstrated in neurodegenerating neurons in AD [49], and thus, its increased expression can be viewed as an attempt to compensate for possible oxidant-induced inactivation of this enzyme.

In conclusion, patients affected by MD are under condition of systemic oxidative stress and the induction of vitagenes Hsp70, is a maintained response in counteracting the intracellular pro-oxidant status. The search for novel and more potent inducers of vitagenes will facilitate the development of pharmacological strategies to increase the intrinsic capacity of vulnerable ganglion cells to maximize antidegenerative mechanisms, such as stress response and thus cytoprotection [11, 26, 32].

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