Neuroscience Letters 606 (2015) 188–193

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research paper

Metabolomic profiling in inner ear fluid by gas chromatography/mass spectrometry in guinea pig cochlea

Takeshi Fujita[a], Daisuke Yamashita[a,*], Yasuhiro Irino[b], Junko Kitamoto[a], Yuriko Fukuda[a], Go Inokuchi[a], Shingo Hasegawa[a], Naoki Otsuki[a], Masaru Yoshida[b,c], Ken-ichi Nibu[a]

[a] Department of Otolaryngology-Head and Neck Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
[b] The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
[c] Division of Metabolomics Research, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

HIGHLIGHTS

- We identified 77 kinds of metabolites in inner ear fluid by metabolome analysis.
- Six metabolites were more abundant in inner ear fluid than in plasma.
- Nine metabolites were less abundant in inner ear fluid than in plasma.
- The levels of 10 metabolites in inner ear fluid changed after loud noise exposure.

ARTICLE INFO

Article history:
Received 16 May 2015
Received in revised form 12 August 2015
Accepted 1 September 2015
Available online 3 September 2015

Keywords:
Metabolomics
GC/MS
Perilymph fluid
Inner ear
Cochlea
Noise-induced hearing loss

ABSTRACT

The composition and homeostasis of inner ear fluids are important in hearing function. The purpose of this study was to perform metabolomic analysis of the inner ear fluid in guinea pig cochlea, which has not been previously reported in literature, using gas chromatography/mass spectrometry (GC/MS). Seventy-seven kinds of metabolites were detected in the inner ear fluid. Six metabolites, ascorbic acid, fructose, galactosamine, inositol, pyruvate + oxaloacetic acid, and *meso*-erythritol, were significantly more abundant, and nine metabolites, phosphate, valine, glycine, glycerol, ornithine, glucose, citric acid + isocitric acid, mannose, and trans-4-hydroxy-L-proline, were less abundant in the inner ear fluid than in plasma. The levels of ten metabolites, 3-hydroxy-butyrate, glycerol, fumaric acid, galactosamine, pyruvate + oxaloacetic acid, phosphate, *meso*-erythritol, citric acid + isocitric acid, mannose, and inositol, in the inner ear fluid significantly changed after loud noise exposure. These observations may help to elucidate various clinical conditions of sensorineural hearing loss, including noise-induced hearing loss.

1. Introduction

The composition and homeostasis of inner ear fluids are of great importance in hearing function. Separation of the endolymph from the perilymph results in an electric potential (i.e., the endocochlear potential) that is fundamental to the transduction processes in the organ of Corti [1]. Moreover, because the organ of Corti lacks its own vascularization, cell metabolism depends on the oxygen and nutrient supply provided by the cochlear fluids. With the exception of the apical surfaces of the hair cells, which are in indirect contact with the endolymph, the cell bodies of the hair cells are fully immersed in the perilymph [2–5]. Hence, the organization of the perilymphatic fluid spaces determines how readily substances can reach different cells in the hearing organ. Moreover, the increasing interest in directly applying therapeutic agents to the perilymphatic fluid of the cochlea (e.g., use of viral vectors for gene therapy) makes characterization of intracochlear fluid communication important [6].

Functional "omics" technologies (e.g., proteomics, transcriptomics, and metabolomics) are gaining increasing importance in the field of medical sciences [7,8]. There have been a few reports on proteomic analysis of perilymph in mouse [9] and human cochlea [10]. Proteomics can provide insight into the understanding of com-

Abbreviations: GC/MS, gas chromatograpy/mass spectrometry; MS-TFA, *N*-methyl-*N*-trimethylsiyl-trifluoroacetamide; SEM, standard error of the mean; dB SPL, decibels sound pressure level.

* Corresponding author. Fax: +81 78 382 6039.
E-mail address: daiy1013@aol.com (D. Yamashita).

http://dx.doi.org/10.1016/j.neulet.2015.09.001
0304-3940/

plex biological systems by analyzing protein expression, function, modifications, and interactions [7]. In contrast, metabolomics has different aspects of advantages with other omics studies. Changes in the levels of metabolites potentially reflect the functional status of a cochlea because their alterations are located downstream of DNA, RNA, and proteins. Therefore, metabolite profile analysis of biofluids may help in the understanding of biological complexity [11,12].

There have been many studies investigating the components of cochlear fluid including the perilymph fluid, from 1970s [13–17]. In the present study, we performed metabolomic analysis using gas chromatography/mass spectrometry (GC/MS) to evaluate differences in the metabolites between the inner ear fluid (mainly perilymph fluid) and blood plasma and the metabolite alterations in the inner ear fluid caused by loud noise exposure.. The present study is, to the best of our knowledge, the first attempt to obtain a detailed profile of the metabolites in inner ear fluid using GC/MS.

2. Material and methods

2.1. Animals and sample preparation

Thirty-five guinea pigs (250–300 g; Hartley males) with normal Preyer's reflex were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee guidelines of Kobe University Graduate School of Medicine (Permit Number: A110107).

The animals were deeply anesthetized with midazolam (10 mg/kg), medetomidine (37.5 mg/kg), and butorphanol tartrate (0.5 mg/kg) intraperitoneally. The animals were then decapitated. The temporal bones were immediately removed. Under a dissecting microscope, round and oval windows were opened, and the lymphatic fluid in the cochlea was gently removed through both the windows. Most of the fluid was thought to be the perilymphatic fluid, but the endolymphatic fluid may have been included. Thus, we treated the fluid simply as "inner ear fluid." The inner ear fluids were obtained and frozen in <5 min after decapitation. Inner ear fluid amounts of approximately 10–15 μl were obtained from one animal (two cochleae), and 5 μl of those amounts were used as one samples, 35). Simultaneously, syringes washed with heparin were used to take blood samples from each guinea pig. The heparinized whole blood was centrifuged, the plasma was transferred to another tube, and 5 μl was used as one sample.

2.2. Experimental protocol

The animals were randomly assigned to a control ($n = 7$) or noise-exposed ($n = 18$) group. The noise-exposed group was divided into two subgroups: the 2 h after noise group (2-h group, $n = 9$) and the 24 h after noise group (24-h group, $n = 9$). The cochleae were removed at 2 h and 24 h, respectively, from the two groups after noise exposure.

We performed two experiments in this study. The first experiment compared the abundance of metabolites in the inner ear fluid and plasma of normal (control) guinea pigs. This experiment was independently repeated four times: first, $n = 5$; second, $n = 3$; third, $n = 3$; and fourth, $n = 6$. The metabolites that were present at statistically significant levels in at least three of the four replicates were selected as the results. The second experiment studied the change in metabolites after noise exposure. This experiment was repeated three times: $n = 3$, each group. The metabolites that were present at statistically significant levels in at least two of the three replicates were selected as the results.

2.3. Noise exposure

The animals were exposed to one-octave-band noise centered at 4 kilohertz (kHz) at a 126-decibel sound pressure level (dB SPL) (permanent threshold shift model) for 2 h in a ventilated sound-exposure chamber. Details of the method of noise exposure have been previously described [18].

2.4. Extraction of intracellular metabolites

2-Isopropyl malic acid (Sigma–Aldrich, MO, USA) was used as the internal standard. Methoxyamine hydrochloride was purchased from Sigma–Aldrich, and *N*-methyl-*N*-trimethylsiyl-trifluoroacetamide (MS-TFA) was purchased from GC Sciences, Inc. (Tokyo, Japan). A 5-μl aliquot of lymph fluid or plasma was added to 45 μl of water and 2.5 μg of 2-isopropylmalic acid as an internal standard to correct the loss of analyte during sample preparation. To extract metabolites, 250 μl of solvent mixture (methanol:chloroform:water; 2.5:1:1) was added and incubated for 30 min at 37 °C. After centrifugation, 225 μl of the supernatant was collected, and 200 μl of water was added and mixed. Further, 250 μl of the supernatant was transferred to a new tube after centrifugation followed by speed vacuum concentration for 20 min. The remaining liquid was placed in a freeze dryer overnight for lyophilization.

2.5. Derivatization of intracellular metabolites for GC/MS analysis

The extracted metabolites were dissolved in 40 μl of 20 mg/ml methoxyamine hydrochloride in pyridine solution and incubated for 90 min at 30 °C. Subsequently, trimethylsilylation was performed by using 20 μl of MS-TFA for derivatization with incubation for 30 min at 37 °C. After incubation, the derivatized samples were centrifuged to remove debris, and the clear liquid was transferred into GC vials for GC/MS analysis.

2.6. GC/MS analytical conditions and data analysis

A GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) system was used with a fused silica capillary column (CP-SIL 8CB low bleed/MS; 30 m × 0.25-mm inner diameter, 0.25-μm film thickness; Agilent Co., CA, USA). The front inlet temperature was 230 °C. The helium gas flow rate through the column was 39.0 cm/s. The column temperature was held at 80 °C for 2 min, increased to 330 °C at 15 °C/min and maintained for 6 min. The injection volume was 1 μl, and samples were injected in split mode with a split ratio of 1:25. The transfer line and ion-source temperatures were 250 °C and 200 °C, respectively. Twenty scans per second were recorded over the mass range of 85–500 m/z using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co.). For data processing, MS data were exported in the netCDF format, and peak detection and alignment were performed using MetAlign software (Wageningen UR, The Netherlands). The resulting data were exported in the CSV-format file and analyzed using in-house analytical software (AI output), as previously described [19,20]. This software enables peak identification and quantification using the in-house metabolites library. For quantification, the peak height of each ion was calculated and normalized to the peak height of 2-isopropylmalic acid used as the internal standard.

2.7. Statistical analysis

A two-tailed *t*-test was used to compare metabolites between the lymphatic fluid and plasma sample in the control group. One-way ANOVA followed by Bonferroni's correction was used to compare the three groups: the control, 2-h group, and 24-h group.