Histone Deacetylase 2 in Sudden Sensorineural Hearing Loss Patients in Response to Intratympanic Methylprednisolone Perfusion

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Abstract

Objective. To evaluate the expression of histone deacetylase 2 (HDAC2) in peripheral blood mononuclear cells (PBMCs) from patients with sudden sensorineural hearing loss (SSNHL) who were refractory to systemic glucocorticoid treatment and to identify the relationship between the level of HDAC2 and glucocorticoid insensitivity.

Study Design. Prospective clinical study.

Setting. This study was conducted in Nanjing Drum Tower Hospital, Nanjing University Medical School.

Subjects and Methods. PBMCs were collected from 42 refractory SSNHL patients. After a 10-day intratympanic methylprednisolone perfusion (IMP) and systemic Ginkgo biloba extract treatment, the SSNHL patients were divided into 2 groups according to their hearing recovery after IMP (IMP sensitive and insensitive). Real-time polymerase chain reaction and HDAC2 protein assays were used to detect the relative expression levels of HDAC2 in PBMCs. The HDAC2 mRNA expression and protein levels in PBMCs collected from 17 volunteers were used as normal HDAC2 reference levels.

Results. Compared with normal reference levels, HDAC2 protein levels were significantly reduced, while the HDAC2 mRNA expression was much higher in all refractory SSNHL patients before IMP. HDAC2 mRNA expression and HDAC2 protein levels were significantly elevated in the IMP-sensitive group, while no change was observed in the IMP-insensitive group after IMP plus systemic antioxidant treatment.

Conclusions. Reduced HDAC2 protein levels may be 1 of the mechanistic underpinnings of corticosteroid insensitivity in refractory SSNHL patients. IMP can increase HDAC2 protein levels and the expression of HDAC2 mRNA in IMP-sensitive patients. HDAC2 protein levels might be regulated through posttranslational modifications.

Keywords
sudden sensorineural hearing loss, glucocorticoid resistance, histone deacetylase 2, posttranslational modification
nucleus. In the nucleus, the GC-GR complex regulates the expression of inflammatory and anti-inflammatory genes.\textsuperscript{10} In this process, histone deacetylase 2 (HDAC2) is recruited to deacetylate the inflammatory genes to inhibit their expression. HDAC2 can interact with various inflammatory factors, such as activator protein 1 and nuclear factor κB, to decrease the expression of inflammatory genes.\textsuperscript{10,11} Some researchers believe that increased expression of inflammatory genes is mediated by increased acetylation of core histones, which is caused by the reduction of HDAC.\textsuperscript{10,11} Reduced HDAC activity has been associated with inflammation in Wegener’s granulomatosis, chronic obstructive pulmonary disease, and asthma.\textsuperscript{12,13} Reduced HDAC2 is also 1 of the mechanisms of GC resistance in patients with asthma and chronic obstructive pulmonary diseases.\textsuperscript{10,11}

HDACs are grouped into 4 classes based on their gene similarity. HDAC2 belongs to class I,\textsuperscript{14,15} which is located in the nucleus and ubiquitously distributed in almost all tissues in the body, including the cochlea.\textsuperscript{15,16} One of the proteins regulated by HDAC2 activity is GR. We previously demonstrated that measurement of GR levels in peripheral blood mononuclear cells (PBMCs) can be used as an indicator of GR levels in the cochlea of guinea pigs.\textsuperscript{17} In this study, we examined HDAC2 protein levels in PBMCs in SSNHL patients who did not respond to conventional therapy (systemic GCs and vasodilators) at our institution and compared those patients who did respond to subsequent salvage therapy with those who did not.

### Methods

**Recruitment of Patients and Intratympanic Methylprednisolone Perfusion Treatment**

The study protocols and informed consent form were approved by the ethics committee of Nanjing Drum Tower Hospital, Nanjing University. Forty-two patients (18-65 years old) were recruited from January 2013 to June 2014. SSNHL is defined as a rapid hearing decline at least 30 dB in \( \geq 3 \) contiguous frequencies in <3 days without any identifiable causes.\textsuperscript{2,4} In this study, all patients had severe hearing loss (PTA at 0.5-4 kHz >60 dB) in the affected ear at the end of the conventional therapeutic regimen at our institution (Table 1). Structural diseases, such as acoustic neuroma and stroke, were ruled out by head and neurologic examination and magnetic resonance imaging. No patient had a history of ear disease or family history of hearing loss. All patients had failed to respond (PTA improvement <15 dB) to a minimum 10-day conventional therapy at our institution, which included systemic GCs (dexamethasone, 2.5-10 mg/d for 3-4 days, intravenously; or methylprednisolone, 20-80 mg/d for 3-4 days, intravenously) and vasodilators (Ginkgo biloba extract injection, 20 mL/d for 10 days, intravenously; Dr Willmar Schwabe, GmbH & Co KG, Essen, Germany), alprostadil injection (10 \( \mu \)g/d for 10 days, intravenously; Beijing Tide Pharmaceutical Co Ltd, Beijing, China), or vinpocetine (20 mg/d for 10 days; Henan Runhong Pharmaceutical Co Ltd, Zhengzhou, China). Eight cases also received hyperbaric oxygen therapy. Participants were all followed up for 3 months. Seventeen volunteers with normal audiograms were enrolled in the study to obtain normal HDAC2 reference levels in PBMCs.

Each SSNHL patient received a 0.5-mL sterile aqueous suspension of methylprednisolone (Pfizer Inc, New York, New York) in a concentration of 40 mg/mL by intratympanic methylprednisolone perfusion (IMP) through a microcatheter once a day for 10 consecutive days.\textsuperscript{9} Each patient also received Ginkgo biloba extract (EGB761, an antioxidant, 105 mg/d for 10 days) and monosialotetrahexosylganglioside sodium (a neurotrophic drug for repairing injured nerves, 40 mg/d for 10 days; Qilu Pharmaceutical Co Ltd, Jinan, China) by intravenous administration. The PTA was tested before IMP as well as the next day after the 10-day IMP treatment. Hearing tests were also performed every 2 to 4 weeks within a 3-month follow-up period. The word recognition test was not performed in the present study due to the current lack of Chinese standard word recognition tables and corresponding word recognition scores. The patients were assigned to 2 groups according to their PTA (0.25-8 kHz) gain 3 months after SSNHL onset: an IMP-sensitive (IMPS) group (\( \geq 15 \) dB), who was sensitive to IMP treatment, and an IMP-insensitive (IMPI) group (\(<15 \) dB), who was insensitive to IMP treatment.

**Isolation of PBMCs**

PBMCs were collected from refractory SSNHL patients before IMP and the last day of 10-day IMP, isolated by Ficoll according to the manufacturer’s instructions (Tian Jin Hao Yang Biological Manufacture Co Ltd, Tianjin, China), and then stored at \(-80^\circ\)C until RNA and protein extraction.

**Total RNA and Protein Extraction from Nuclear and Cytosolic Fractions for Analysis of HDAC2 Levels**

To extract total RNA, the frozen PBMCs were resuspended by adding 1 mL of TRIzol (Invitrogen, Waltham, Massachusetts), incubated for 15 minutes at room temperature, and vortexed briefly after adding 200 \( \mu \)L of chloroform. The mixture was centrifuged, and the aqueous phase was transferred to an

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### Table 1. Demographic Data of Patients with SSNHL (N = 42).

<table>
<thead>
<tr>
<th>Patients, n or Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male:female</td>
</tr>
<tr>
<td>Average age, y</td>
</tr>
<tr>
<td>Ear side, left:right</td>
</tr>
<tr>
<td>Time from onset to IMP, d</td>
</tr>
<tr>
<td>PTA before IMP, 0.25-8 kHz</td>
</tr>
<tr>
<td>Vertigo</td>
</tr>
<tr>
<td>Audiogram shape</td>
</tr>
<tr>
<td>Total deafness</td>
</tr>
</tbody>
</table>

Abbreviations: IMP, intratympanic methylprednisolone perfusion; PTA, pure tone average; SSNHL, sudden sensorineural hearing loss.
mRNA expression was expressed as 2^{-\Delta Ct} value of GAPDH for each sample. HDAC2 was obtained by subtracting the average Ct value of HDAC2 from the fixed detectable threshold—were read, and the number when the amount of amplified copies reaches a single-stranded cDNA through the PrimeScript RT-PCR Kit (Takara, Kusatsu, Japan) according to the manufacturer’s protocol. The nuclear protein fraction was used for measuring HDAC2 level.

### Quantitative Real-Time Polymerase Chain Reaction and HDAC2 Level

HDAC2 mRNA expression was determined by quantitative real-time polymerase chain reaction (PCR) with SYBR Green PCR Master Mix Reagent (Applied Biosystems, Foster City, California). Reverse transcription of RNA was carried out with the PrimeScript RT-PCR Kit (Takara) according to the manufacturer’s protocol. The Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, California) was used to perform real-time PCR. The PCR primers were designed by PrimerBank:

- **HDAC2:** forward—5’–ATGGCGTACAGTGCAAGGAGG–3’ and reverse—5’–TGGCGATTCTATG–3’
- **GAPDH:** forward—5’–GCACCGTCAAGGCTGAGA–3’ and reverse—5’–TGGTGAAGACGCCAG–3’

The thermal cycle conditions included a 30-second initial setup at 95°C, followed by 40 cycles of 5-second denaturing at 95°C and 34-second annealing/extension at 60°C. When the cycles finished, the threshold cycle (Ct) values of HDAC2 and GAPDH—which indicate the fractional cycle number when the amount of amplified copies reaches a fixed detectable threshold—were read, and \(\Delta Ct\) was calculated by subtracting the average Ct value of HDAC2 from the average Ct value of GAPDH for each sample. HDAC2 mRNA expression was expressed as \(2^{-\Delta Ct}\).

HDAC2 protein levels were measured with a commercially available HDAC2 assay kit (EpiQuik, Farmingdale, New York). Nuclear protein was diluted to a concentration of 0.8 \(\mu\)g/\(\mu\)L, and 10 \(\mu\)L of the solution was added into the central area of each well. The strip wells were incubated at 37°C for at least 90 minutes to completely dry the wells. Optical density (OD) values were recorded on the microplate reader at 450 nm and were used to calculate relative HDAC2 protein levels for each patient.

### Data Analysis

All statistical analyses were conducted with SPSS 17.0. The data were expressed as the mean ± SD. Independent-sample t test was used to determine if there were statistically significant differences between the IMPS and IMPI groups, refractory SSNLH patients, and healthy people. The comparisons of PTA, HDAC2 mRNA expression, and HDAC2 levels before and after therapy were analyzed by paired t test. \(P < .05\) was considered significant.

### Results

#### Audiometric Outcomes

The PTA of each patient was evaluated at 24 hours and 2 weeks after the 10-day IMP treatment, as well as at 1, 2, and 3 months of follow-up periods. Hearing improvement was defined as a decrease of PTA (0.25-8 kHz) \(\geq 15\) dB. The SSNHL patients were assigned to 2 groups according to their hearing recovery: an IMPS group (PTA gain \(\geq 15\) dB) and an IMPI group (PTA gain <15 dB). There were 22 patients in the IMPS group and 20 in the IMPI group (Table 2).

Before IMP, the average PTA was 82.20 ± 13.93 dB in the IMPS group and 84.10 ± 12.29 dB in the IMPI group without significant difference (\(t = 0.466, P = .644\)). After IMP, significant hearing improvement was observed in the IMPS group (PTA = 49.42 ± 19.64 dB, \(P = .000\)) but not in the IMPI group (PTA = 83.17 ± 12.99 dB, \(P = .573\); Table 2). The average thresholds at each frequency of the 2 groups before and after IMP are shown in Figure 1. The number of patients before IMP whose PTA (0.5-3 kHz; 3-8 kHz) threshold was an average of 2 kHz and 4 kHz fell into each bin is shown in Figure 2a. The number of patients who had a change in hearing in PTA (0.5-3 kHz) is shown in Figure 2b. These data are presented according to the American Academy of Otolaryngology—Head and Neck Surgery’s minimum reporting standard.18 These results

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**Table 2. PTA before and after Treatment in IMPS and IMPI Groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>t</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMPS (n = 22)</td>
<td>82.20 ± 13.93</td>
<td>49.42 ± 19.64</td>
<td>11.99</td>
<td>.000</td>
</tr>
<tr>
<td>IMPI (n = 20)</td>
<td>84.10 ± 12.29</td>
<td>83.17 ± 12.99</td>
<td>0.573</td>
<td>.573</td>
</tr>
<tr>
<td>t</td>
<td>0.466</td>
<td>6.624</td>
<td>.644</td>
<td>.000</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviations: IMPI, intratympanic methylprednisolone perfusion insensitive; IMPS, intratympanic methylprednisolone perfusion sensitive; PTA, pure tone average.
confirm our previous report that about half of refractory SSNHL patients were responsive to the IMP treatment.9

**Increased HDAC2 Level and HDAC2 mRNA Expression**

Before IMP, HDAC2 protein level was $0.672 \pm 0.034$ (OD value) in refractory SSNHL patients, much lower than the normal reference (OD value = $0.755 \pm 0.048$, $t = 7.583$, $P = .000$; Figure 3). As shown in Figure 4, HDAC2 mRNA expression was $0.084 \pm 0.034$ ($2^{-\Delta Ct}$) in refractory SSNHL patients, much higher than normal reference ($0.016 \pm 0.005$, $2^{-\Delta Ct}$, $t = 12.25$, $P = .000$).

HDAC2 protein levels were $0.677 \pm 0.026$ (OD value) in the IMPS group and $0.666 \pm 0.041$ (OD value) in the IMPI group, revealing that there was no significant difference between the 2 groups ($t = 0.963$, $P = .343$; Figure 5). HDAC2 mRNA expression was $0.080 \pm 0.027$ ($2^{-\Delta Ct}$) in the IMPS group and $0.087 \pm 0.042$ ($2^{-\Delta Ct}$) in the IMPI group, also indicative of no significant difference between the 2 groups ($t = 0.652$, $P = .518$; Figure 6). However, it is unclear whether the previous systemic GC treatment changed HDAC2 protein levels, since all patients received systemic GCs and vasodilators treatment before IMP + Ginkgo biloba extract treatments. After IMP, HDAC2 mRNA expression ($0.149 \pm 0.027$, $2^{-\Delta Ct}$, $t = 6.35$, $P = .000$) and
HDAC2 protein levels (OD value = 0.717 ± 0.02, \( t = 2.86, P = .009 \)) in the IMPS group were significantly increased (Figures 4 and 6). However, HDAC2 protein levels (OD value = 0.656 ± 0.04, \( t = 0.847, P = .407 \)) and HDAC2 mRNA expression (0.093 ± 0.04, 2\(^{-\Delta\Delta C_t}, t = 0.391, P = .700 \)) did not change in the IMPI group (Figures 4 and 6).

Discussion

The present study demonstrates that about 52% of refractory SSNHL patients were responsive to IMP + antioxidant treatment, which is consistent with our previous report.\(^9\) These results and previous reports indicate that most refractory SSNHL patients who failed systemic treatments can still be treatable. Local GC treatment can reach higher GC concentration in the inner ear than that of systemic treatment.\(^{19}\) GCs can suppress inflammation in the inner ear, increase cochlear blood flow to improve microcirculation, and maintain the ionic balance of perilymph and endolymph.\(^3,4\) Results in this study also indicate that GC + antioxidant treatment can increase HDAC2 protein levels and its mRNA expression in the IMPS group. GC mediates its effects through binding to the cytoplasmic GR and then translocating into the nucleus to suppress the expression of inflammatory genes and enhance the expression of anti-inflammatory genes.\(^{20}\) It is thought that acetylation of core histones induces topologic relaxation of DNA, which allows transcription factors to promote gene expression. GC can reverse this process by recruiting HDAC2 to inflammatory genes and repress their expression.\(^{20}\) HDAC2 can deacetylate GR after GC binding and allow it to access to the p65–nuclear factor κB activated complex and subsequently suppress inflammatory gene expression. Consistent with our results, reduction of HDAC2 expression has been shown to induce GC insensitivity.\(^{24}\) In contrast, overexpression of HDAC2 can restore GC functions.\(^{22,23}\)

In the present study, before IMP, all refractory SSNHL patients had lower HDAC2 protein levels but higher mRNA expression as compared with healthy people. The disagreement between HDAC2 protein level and mRNA expression might be the consequence of \( \geq 1 \) pathologic processes associated with SSNHL, posttranslational regulation of HDAC2, or the therapeutic effect of conventional therapy at our institution. Consistent with this rationale, previous studies demonstrated that HDAC2 levels can be reduced by posttranslational modifications, such as nitration and oxidation.\(^{11}\) Thus, the reduced HDAC2 protein levels observed in our study may be a consequence of pathologic changes associated with SSNHL, such as oxidative stress and/or inflammation in the cochlea.\(^{24}\) Progressive oxidative stress can significantly reduce the activity of HDAC2 leading to GC insensitivity.\(^{10,22,26}\) Similarly, the phosphoinositide 3-kinase (PI3K)/Akt pathway is sensitive to oxidative stress and thus may promote phosphorylation and inactivation of HDAC2. In contrast, LY-294002—a nonselective PI3K inhibitor or knockdown of PI3Kd by RNA interference—has been shown to reverse steroid insensitivity.\(^{22}\) Because all patients were treated with systemic GCs in the present study, future research should investigate the HDAC2 status in SSNHL patients without any treatment.

However, approximately 50% of SSNHL patients were unresponsive to local GC + systemic antioxidant treatment. These patients did not show any changes in either HDAC2 protein levels or mRNA expression. These results suggest that inherently low HDAC2 levels in a subset of SSNHL patients may play a fundamental mechanistic role in GC insensitivity, augmenting a growing list of inflammatory diseases in which unresponsive patients of this type have been described.\(^{10,11,16}\) Currently, a great deal of emphasis is placed on the relationship between reduction of HDAC2
activity and GC insensitivity in the areas of chronic obstructive pulmonary disease and allergic asthma. To our knowledge, there has been no report of a study in which HDAC2 activity was monitored in SSNHL patients. In the present study, we showed that HDAC2 protein levels were reduced in PMBCs in SSNHL patients who had failed to positively respond to ≥10 days of conventional therapy at our institution. This finding is consistent with other studies in chronic obstructive pulmonary disease and allergic asthma. Intratympanic perfusion of GC improved the functional levels of HDAC2 in PBMCs, which may indirectly reflect the level of HDAC2 enzymatic activity in the inner ear. We believe that the restoration of HDAC2 function—and, thus, sensitivity to GC in the inner ear—may directly contribute to the prognosis for hearing recovery.

In conclusion, reduced HDAC2 is associated with GC resistance. Local GC and systemic antioxidant treatment can restore HDAC2 protein levels to treat refractory SSNHL.

Acknowledgments

We appreciate Dr Matthew B. West’s invaluable comments in preparing the manuscript.

Author Contributions

Jie Hou, drafted the manuscript, analyzed and interpreted the data, revised the manuscript, approved the final version, agreed to be accountable for all aspects of the work; Wandong She, planned the study, interpreted the data, revised the manuscript critically, approved the final version, agreed to be accountable for all aspects of the work; Xiaoping Du, designed the work partially, interpreted the data, revised the manuscript critically, approved the final version, agreed to be accountable for all aspects of the work; Yanhong Dai, collected clinical data, reviewed the manuscript, approved the final version, agreed to be accountable for all aspects of the work; Lisheng Xie, collected and interpreted the experimental data partially, reviewed the manuscript, approved the final version, agreed to be accountable for all aspects of the work; Qiongqiong Zhou, interpreted the data, reviewed the manuscript, approved the final version, agreed to be accountable for all aspects of the work.

Disclosures

Competing interests: None.

Sponsorships: None.

Funding source: This study was supported by a grant (81271074) from the National Natural Science Funds of China; a grant (BL2014002) for clinical medicine from the science and technology department of Jiangsu Province, China; a grant (WSN-009) from the Six Talent Peaks Project of Jiangsu Province, China; a grant (2012sd311038) for international joint research from the science and technology department of Nanjing, Jiangsu Province, China.

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