INTRODUCTION

Glucocorticoids (GCs) are currently the major drug in the treatment of certain types of inner ear disorders, such as sudden sensorineural hearing loss (SSNHL), noise-induced hearing loss, Meniere’s disease and cisplatin-induced ototoxicity [1,2]. They are particularly recommended as the first line treatment for SSNHL in clinical practice guidelines in many countries [3,4]. It has been reported that GC can decrease inflammation, increase the expression of antioxidant enzymes, and reduce apoptosis induced by ototoxicity and SSNHL in the cochlea [2,5].

GCs bind to glucocorticoid receptors (GRs) to provide anti-inflammatory and anti-apoptotic functions in the target tissues. GRs belong to the nuclear hormone receptor superfamily and contain three major functional domains: a N-terminal transactivation domain, a central DNA-binding domain, and a C-termina-
nal ligand-binding domain [6]. The GRs have two major iso-
forms, GRα and GRβ, which are different in the final coding
exon. GRα is thought to be the main mediator of GC
action while GRβ is considered as an antagonist of GRα [7]. GRα and
GRβ are ligand-dependent transcription factors and expressed in
most cell types, however, GRβ is generally expressed at low lev-
els compared to GRα [7]. In the human inner ear, the distribu-
tion of GRs is widespread, and the highest concentration is ob-
served in the spiral ligament [8].

Although most SSNHL patients respond well to GC treatment,
about 20% of SSNHL patients show little or no response to GC
treatment, i.e., GC-resistance [9]. Mechanisms of GC-resistance
are still unknown. Recent studies suggest that changes in cellular
micro-environment, induction of cytokines as a result of chronic
inflammation, hypoxia, oxidative stress, allergen exposure and
serum-derived factors may contribute to the development of GC
resistance [10]. Beside these mechanisms, an accumulating body
evidence indicates that posttranslational modification of GRs
and reduced histone deacetylase 2 (HDAC2) activity are also in-
volved in steroid resistance [11]. Some studies indicate that up-
regulation of GRα and downregulation of GRβ can improve ste-
roid sensitivity [12,13]. This model is supported by observations
that histone deacetylases are recruited by GRs for transrepres-
sion [14]. A recent study also suggests that GRα increases HDAC2
promoter activity, while GRβ abolishes this effect in a dose-de-
pendent manner [11]. Our previous study indicated that reduced
HDAC2 protein level may be one of the mechanisms of GC in-
sensitivity in patients with refractory SSNHL [15]. In this study,
we further explored the roles of HDAC2 and two isoforms of
GR in GC sensitivity in patients with severe and profound SSNHL.

MATERIALS AND METHODS

Subjects

The study was conducted at The Affiliated Jiangyin Hospital of
Southeast University Medical School from January 2015 to Oc-
tober 2017. The study protocols were approved by the hospital
Ethics Committee (No. ChiCTR-BOC-16008999).

Fifty-five SSNHL patients with severe or profound hearing
loss (pure tone audiometry [PTA] at 0.5–4 kHz ≥61 dB within
72 hours of onset according to American Academy of Otolaryn-
gology-Head and Neck Surgery guidelines) [3] were included in
the study. Exclusion criteria included middle or external ear dis-
eases or middle ear surgery history, retrocochlear diseases, ma-
lignancy or autoimmune diseases or head trauma history, or a
history of asthma or chronic obstructive pulmonary disease, or
a family history of hearing loss. Subjects pretreated with GCs or
other medicines before this study were also excluded. Informed
consent documentation was obtained from all patients before
treatment.

All patients received a conventional therapy, including sys-
temic GC (methylprednisolone sodium succinate, 80 mg/day for
4 days, then 40 mg/day for 3 days, and 20 mg/day for another 3
days; Pfizer Manufacturing Belgium NV, Puurs, Belgium), anti-
oxidant (105 mg/day for 10 days, Ginkgo biloba extract injec-
tion; Dr. Willmar Schwabe GmbH & Co. KG, Essen, Germany),
and mecobalamin injection (500 μg/day for 10 days; Yangtze
River Pharmaceutical Group, Nanjing Hailing Pharmaceutical,
Nanjing, China). PTA was measured before and 24 hours after
the 10-day conventional treatment, as well as during a 3-month
follow-up period. According to hearing improvement at 0.25–
8 kHz, patients were assigned into two groups: a GC-sensitive
(group hearing improvement ≥15 dB, GCS group) and a GC-
resistant group (hearing improvement <15 dB, GCR group) ac-
cording to the guideline published by Editorial Board of Chinese
Journal of Otorhinolaryngology-Head & Neck Surgery and Chi-
nese Medical Association [16]. Twenty healthy volunteers without
hearing loss were also enrolled in the study as a normal reference
group (NR group) to obtain the normal reference levels of GRs
and HDAC2 in peripheral blood mononuclear cells (PBMCs).

Preparation of PBMCs

Peripheral blood was collected before and 24 hours after the 10-
day conventional GC treatment. PBMCs were isolated by the
Ficoll-Paque Plus density-gradient method (GE Healthcare Bio-
sciences, Uppsala, Sweden) and stored at –80°C until RNA and
protein extractions (detailed below).

Quantitative real-time polymerase chain reaction

The mRNA levels of GRα, GRβ and HDAC2 were measured by
quantitative real-time polymerase chain reaction (qPCR). Total
RNA was isolated from PBMCs by adding 1 mL Trizol (Invitro-
gen, Waltham, MA, USA) and then 200 μL chloroform. RNA
was used to synthesize single-stranded cDNA by the Prime
Script RT-PCR Kit (Takara, Kusatsu, Japan) according to the
manufacturer’s protocol. qPCR was performed with SYBR
Green PCR Master Mix Reagent (Takara). The Applied Biosys-
tems 7500 real-time PCR system (Applied Biosystems, Foster
City, CA, USA) was used to perform real-time PCR. The primers
were designed by Primer Bank (Table 1). The thermal cycle con-
ditions were consisted of one 30-second cycle of predenatur-
atation at 95°C followed by 40 cycles of 5-second denaturation at
Table 1. Primers used for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGRα upstream primer</td>
<td>5′-GGAGGACCTCCAGCCAGA-3′</td>
</tr>
<tr>
<td>hGRα downstream primer</td>
<td>5′-CTGAGTGATGGTCTGTGATA-3′</td>
</tr>
<tr>
<td>GRβ upstream primer</td>
<td>5′-CCTAGGACCTGGTCTGAGAC-3′</td>
</tr>
<tr>
<td>GRβ downstream primer</td>
<td>5′-CCACGTATCTTAAAGGGCC-3′</td>
</tr>
<tr>
<td>HDAC2 upstream primer</td>
<td>5′-ATGCGCTACAGTCAAGGAGG-3′</td>
</tr>
<tr>
<td>HDAC2 downstream primer</td>
<td>5′-GACGGATCTATGAGGCTTCA-3′</td>
</tr>
<tr>
<td>GAPDH upstream primer</td>
<td>5′-CTGGGCTACACTGAGCACC-3′</td>
</tr>
<tr>
<td>GAPDH downstream primer</td>
<td>5′-AACGACCTGGTCAAGAGCACC-3′</td>
</tr>
</tbody>
</table>

qPCR, quantitative real-time polymerase chain reaction; hGR, human glucocorticoid receptor; GRβ, GRβ1; HDAC2, histone deacetylase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

95°C and 30-second annealing/elongation at 60°C. Melt curve analysis was performed to ensure the amplification of a single product. Target mRNA expression levels were calculated by the 2-ΔΔCt method [17].

Western blot analysis

The protein levels of GRα, GRβ, and HDAC2 were measured by Western blot. To extract total protein, cells were homogenized in ice-cold radio immunoprecipitation assay Lysis Buffer (Beyotime, Nanjing, China) containing 1 mM phenylmethylsulfonyl fluoride. Debris was removed by centrifuging at 13,000 × g for 5 minutes at 4°C. Protein concentrations were determined by using the BCA protein assay kit (Beyotime, Shanghai, China). Thirty-five micrograms of total protein from each sample were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane. Polyclonal rabbit anti-GRα antibody (1:500, ab3580; Abcam, Cambridge, UK), polyclonal rabbit anti-GRβ antibody (1:500, ab3581; Abcam), or monoclonal rabbit anti-HDAC2 antibody (1:1,000, Abcam, Cambridge, UK) served as a loading control. Developed X-ray films were scanned, and densitometries of the immunolabeled bands were quantified by using Image J software ver. 1.34 (http://rsb.info.nih.gov/ij/).

Statistical analysis

All statistical analyses were performed by using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The qPCR and immunoblot data were presented as the mean ± standard deviation. Paired samples t-test was used to analyze PTA, mRNA expression and protein levels of Grα, GRβ, and HDAC2 before and after GC treatment. Independent sample t-test, one-way analysis of variance test and t-test, one-way analysis of variance test and t-test were used to analyze PTA, mRNA expression and protein levels of Grα, GRβ, and HDAC2 before and after GC treatment. Independent sample t-test, one-way analysis of variance test and t-test were used to determine statistical difference between two groups. P-values less than 0.05 were considered statistically significant.

RESULTS

Significant hearing recovery in the GCS group

All SSNHL patients in this study were first diagnosed. Ten SSNHL patients had severe hearing loss (PTA, 61–80 dB at 0.25–4 kHz), and 45 patients (approximately 81.82%) had profound hearing loss (PTA >80 dB at 0.25–4 kHz) before treatment. According to the recovery of PTA at 3-month follow-up, 37 patients were assigned into the GCS group (hearing improvement >15 dB), and 18 patients into the GCR group (hearing improvement >15 dB). The patients’ sex, the side of affected ears and the shape of hearing loss (PTA ≤80 dB) before treatment. According to the recovery of PTA at 3-month follow-up, 37 patients were assigned into the GCS group (hearing improvement >15 dB), and 18 patients into the GCR group (hearing improvement >15 dB). The patients’ sex, the side of affected ears and the shape of hearing loss (PTA ≤80 dB). No significant differences were observed in the number of patients with profound hearing loss in the two groups (94.4% in the GCR group; 75.7% in the GCS group; P=0.09) (Table 3). Significant hearing improvement was only observed in the GCS group (PTA, 48.29±15.30 dB; P<0.001), not in the GCR group (PTA, 87.72±11.28 dB; P=0.07) after treatment.

Increased mRNA expression of GRα and HDAC2 in the GCS group

Qualitative evaluations of qPCR amplifications of GRα, GRβ, and HDAC2 mRNA in PBMCs revealed single melting curve peaks for each amplicon and single electrophoretic bands for each reaction, corresponding to the predicted size of each product. GRα and HDAC2 mRNAs were detected in all samples. However, GRβ mRNA was only detected in 87 of the 130 sam-

Table 2. Clinical characteristics of patients with SSNHL (n=55)

<table>
<thead>
<tr>
<th>Variable</th>
<th>GCS group</th>
<th>GCR group</th>
<th>Hearing improvement rate (%)</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
<td>63.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Side of lesion</td>
<td>Left</td>
<td>Right</td>
<td>62.5</td>
<td>0.37</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>Yes</td>
<td>No</td>
<td>27.3</td>
<td>0.09</td>
</tr>
<tr>
<td>Severe&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>1</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td>Profound&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28</td>
<td>17</td>
<td>62.2</td>
<td></td>
</tr>
</tbody>
</table>

SSNHL, sudden sensorineural hearing loss; GCS, glucocorticoid-sensitive; GCR, glucocorticoid-resistant; PTA, pure tone audiometry.

<sup>a</sup>Comparison between GCS and GCR groups using exact probability in fourfold table. <sup>b</sup>PTA at 0.25–8 kHz ≤80 dB. <sup>c</sup>PTA at 0.25–8 kHz >80 dB.
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Table 3. Hearing loss severity in GCS and GCR groups

<table>
<thead>
<tr>
<th>Initial hearing loss severity</th>
<th>GCS group</th>
<th>GCR group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients (%)</td>
<td>PTA gain after treatment (dB, 0.25–8 kHz)</td>
</tr>
<tr>
<td>Severe HL (61–80 dB)</td>
<td>9 (24.3)</td>
<td>27.63±10.01</td>
</tr>
<tr>
<td>Profound HL (&gt;80 dB)</td>
<td>28 (75.7)</td>
<td>39.70±15.28</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. GCS, glucocorticoid-sensitive; GCR, glucocorticoid-resistant; PTA, pure tone average.

Fig. 1. Glucocorticoid receptor α (GRα) (A) and GRβ mRNA (B) relative expression in the normal reference (NR) group and sudden sensorineural hearing loss patients before treatment. There were no significant differences between groups (all \( P > 0.05 \)). Comparison of GRα (C) and GRβ mRNA (D) relative expression in the glucocorticoid-sensitive (GCS) group and the glucocorticoid-resistant (GCR) group before and after glucocorticoid (GC) treatment. After GC treatment, GRα mRNA expression significantly increased in the GCS group (\( P < 0.001 \)), while no significant change was observed in the GCR group (\( P = 0.91 \)). After GC treatment, slightly increased GRβ mRNA expression was observed in the GCS and the GCR groups, however, there were no significant differences between groups (all \( P > 0.05 \)). (A, C) Comparison between groups using one-way analysis of variance test. (C, D) Comparison before and after treatment using paired-sample test.

Examples (pretreatment: 31 samples in GCS, 12 samples in GCR groups, 12 samples in NR group; posttreatment: 21 samples in GCS and 11 samples in GCR groups), probably due to a very low-level of GRβ mRNA in these samples. Eventually, both pre- and posttreatment GRβ expression was detected in 16 GCS subjects and 8 GCR subjects and the results were used for a pair comparison. GRβ mRNA was detected in 12 NR subjects.

Quantitative PCR evaluations revealed that, before GC treatment, all SSNHL patients had equivalent levels of GRα, GRβ, and HDAC2 mRNA expressions as the NR group (all \( P > 0.05 \)) (Fig. 1A, B, and 2A).

After GC treatment, GRα and HDAC2 mRNA expression levels were all increased in the GCS group (GRα, \( P < 0.001 \); HDAC2, \( P < 0.001 \)) (Fig. 1C, 2B). However, such increase was not observed in the GCR group (GRα, \( P = 0.91 \); HDAC2, \( P = 0.52 \)) (Fig. 1C, 2B). There was a slightly increased GRβ mRNA expression in both the GCS and GCR groups after treatment compared to the levels before treatment, but no significant differences were observed (all \( P > 0.05 \)) (Fig. 1D).
Increased protein levels of GRα and HDAC2 after GC treatment

The relative protein levels of GRα, GRβ, and HDAC2 were calculated and normalized against the internal β-actin standard among 20 NR, 37 GCS, and 18 GCR subjects. Before GC treatment, significantly lower GRα protein levels were observed in the GCR and GCS groups compared to the NR group (0.56 ± 0.09 in the GCR group, 0.59 ± 0.10 in the GCR group, 0.68 ± 0.09 in the NR group) (Fig. 3, 4A). The levels of GRα were significantly increased in the GCS group after GC treatment (P < 0.001), while no change was observed in the GCR group (P = 0.60) (Fig. 3, 4B). No GRβ protein was detected in any sample we tested.

Fig. 3. Western blotting for glucocorticoid receptor α (GRα) (86 kD) and histone deacetylase 2 (HDAC2; 55 kD) in the normal reference (NR), glucocorticoid-sensitive (GCS) and glucocorticoid-resistant (GCR) groups. Each lane represents one sample from each group. *Before glucocorticoid treatment. **After glucocorticoid treatment.

Fig. 4. (A) Glucocorticoid receptor α (GRα) protein in the normal reference (NR) group and sudden sensorineural hearing loss (SSNHL) patients before glucocorticoid (GC) treatment. Prior to treatment, significantly lower GRα protein levels were observed in the glucocorticoid-sensitive (GCS) and glucocorticoid-resistant (GCR) groups compared to the NR group (P = 0.001 or P < 0.05), with the lowest GRα protein levels observed in the GCR group. (B) Comparison of GRα protein level in the GCS and GCR groups before and after GC treatment. After GC treatment, GRα protein levels significantly increased in the GCS group (P < 0.001), while no significant change was observed in the GCR group (P = 0.60). (A) Comparison between groups using one-way analysis of variance test. (B) Comparison before and after treatment using paired-sample test.
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Fig. 5. (A) Histone deacetylase 2 (HDAC2) protein in the normal reference (NR) group and sudden sensorineural hearing loss (SSNHL) patients before glucocorticoid (GC) treatment. Prior to GC treatment, HDAC2 protein levels were significantly lower in SSNHL patients compared to the NR group (P<0.001). (B) Comparison of HDAC2 protein levels in the glucocorticoid-sensitive (GCS) and glucocorticoid-resistant (GCR) groups before and after GC treatment. After glucocorticoid (GC) treatment, HDAC2 protein levels significantly increased in the GCS group (P<0.001), while no significant change was observed in the GCR group (P=0.12). (A) Comparison between groups using one-way analysis of variance test. (B) Comparison before and after treatment using paired-sample test.

Fig. 6. The comparison of the expression levels of glucocorticoid receptor α (GRα) (A, B) and histone deacetylase 2 (HDAC2) (C, D) between the normal reference (NR) group and the glucocorticoid-sensitive (GCS) group after treatment. After glucocorticoid (GC) treatment, the mRNA level of GRα and HDAC2 in GCS group significantly increased and were higher compared with NR group (P<0.001 or P=0.05). The protein level of GRα in GCS group also significantly increased (P<0.05) and were higher compared with NR group. The protein level of HDAC2 also increased after treatment but were still lower compared with NR group (P<0.05). Comparison between groups using independent samples t-test.

As shown in Fig. 5A, HDAC2 protein level was 0.41±0.09 in the GCS group, 0.40±0.08 in the GCR group, much lower than 0.62±0.12 in the NR group before GC treatment. After GC treatment, HDAC2 protein level was significantly increased in the GCS group (P<0.001) (Fig. 3, 5B), while no change was observed in the GCR group (P=0.12) (Fig. 3, 5B).
Comparison of expression levels of GRα and HDAC2 between NR and GCS group after treatment

After GC treatment, the expression levels of GRα and the mRNA level of HDAC2 in GCS group significantly increased and were higher compared with NR group ($P<0.001$ or $P=0.05$) (Figs. 6A-C). The protein level of HDAC2 also increased after treatment but were still lower compared with NR group ($P<0.05$) (Fig. 5B, 6D). So the ability of increased expressions of GR and HDAC2 after GC treatment may be the critical role for GC sensitivity in SSNHL, irrespective to their expression levels compared to NR.

**DISCUSSION**

The molecular mechanisms underlying steroid resistance remain unclear. However, it has been proposed that reduced expression and/or activity of GRs and HDAC2 may play an important role in GC insensitivity [10,13,19]. It has been suggesting that reduced HDAC2 may be caused by increased expression of GRβ in asthma and chronic obstructive pulmonary diseases although conflicting reports are found in literature [11,12,19]. Our previous studies have suggested that reduced HDAC2 is also associated with steroid resistance in refractory SSNHL patients [15]. In the present study, we have extended these evaluations by examining GRα, GRβ, and HDAC2 gene expression and protein levels in PBMCs of patients with severe or profound SSNHL before and after GC treatment. We demonstrated that both the mRNA and protein levels of GRα and HDAC2 were significantly increased in patients who had significant hearing improvement after GC therapy, while no changes were observed in GCR patients. These data suggest that GRα and HDAC2 may be critical factors for mediating, or at the very least predicting, GC sensitivity in SSNHL patients.

In the present study, the GRα and HDAC2 mRNA level before GC treatment was identical in the GC-resistant, GC-sensitive and NR groups while HDAC2 protein levels were significantly lower in all SSNHL patients than the NR group. The discrepancies between mRNA and protein expression levels could be explained by posttranslational regulation [15]. Some factors in the pathogenesis of SSNHL, such as oxidative stress or inflammation, could affect the posttranslational regulation of GR and HDAC2 [20-22]. Lower protein levels of GR and HDAC2 expression might be evoked by a destabilization of mRNA and/or by the induction of ubiquitination and proteasomal degradation [20]. Since both GCS and GCR groups had reduced protein levels of GRα and HDAC2 before treatments, the reduced GRα and HDAC2 could be causative factors of SSNHL as well as the prognostic factors of SSNHL. However, we believe that the reduced GRα and HDAC2 are closely related to GC insensitivity in SSNHL patients [15] as shown in other systems [23]. The potential molecules causing reduction of HDAC2 and GRα in SSNHL may include nitric oxide (NO), free radicals, as well as phosphoinositide-3-kinase (PI3K) δ and erythroid-derived-2 like 2 (Nrf2) that can be activated by oxidative stress [24].

However, immediately after GC therapy, significant elevations in HDAC2 mRNA and protein levels were uniquely observed in the GCS group. These data suggest that no or low response of HDAC2 to steroid treatment in GC-resistant SSNHL patients may play a fundamental mechanistic role in GC insensitivity, which is consistent with our previous report [15]. HDAC2 is a critical component of the GC-GR complex that mediates the transrepression of NF-κB transcriptional activity by deacetylating histones in the proinflammatory genes and by deacetylating GR [19]. In steroid-resistant asthma patients, HDAC2 expression is greatly reduced in alveolar macrophages and PBMCs [25]. The efficacy of GCs was affected when HDAC2 were knockeddown [21]. Both PI3Kδ inhibitors and Nrf2 activators can increase HDAC2 expression and reverse steroid resistant [24]. The potential molecular mechanism of HDAC2 level or activity reduction in SSNHL is that oxidative stress activates phosphoinositide-3-kinase δ, and the latter leads to phosphorylation and inactivation of HDAC2. On the other hand, the reduction of Nrf2 is also linked to reduced HDAC2 expression by preventing HDAC2 deacetylation and activation [24]. Therefore, in light of our current results, the manner in which these factors contextually impact GC resistance in SSNHL patients, bears further evaluation.

Significant elevations in GRα mRNA and protein levels were also observed in the GCS group immediately after GC therapy. These data suggest that, besides HDAC2, low level of GRα in GC-resistant SSNHL patients may also play a fundamental mechanistic role in GC insensitivity. GRα is thought to be the main mediator of GC action [7]. A high GRα level is essential for GC response as demonstrated in various cell lines [26].

The mRNA and protein levels of GRβ were very low or undetectable in PBMCs in all SSNHL patients. Although the GRβ mRNA expression in PBMCs was slightly increased in all patients after GC treatment, no significant difference was observed. These results suggest that GRβ may not be playing a significant role in steroid resistance in SSNHL. Consistent with our results, Hausmann et al. [27] and Butler et al. [12] did not find elevated GRβ mRNA level in PBMCs or lung epithelial cells in steroid-resistant asthma patients. High levels of GRα with very low levels of GRβ were also observed in adult immune thrombocytopenia, suggesting GRβ may not be involved in steroid resistance [13]. Given the substantially higher expression of GRα, GRβ is unlikely to function as a dominant-negative isoform of GRα and likely has little to do with the steroid resistance [12]. Therefore, targeted modulation of GRα levels could be used as a therapeutic strategy to overcome GC resistance [21].

Our results have demonstrated that the relative response of GRα and HDAC2 to steroid treatment may predict the prognosis of hearing improvement in SSNHL patients. Our data have also indicated that GRβ may not play a significant role in steroid resistance in patients with severe SSNHL.
CONFLICT OF INTEREST

No conflict of interest relevant to this article was reported.

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