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Up-regulation of cochlear Hes1 expression in response to noise exposure

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Hes1, a hairy and enhancer of split homolog, negatively regulates inner ear hair cell differentiation. The main objective of this study was to investigate the status of the Hes1 gene in the noise-damaged cochlea in relation to the degree of noise-induced hearing loss (NIHL). Adult albino guinea pigs were exposed to white-band noise (115 dB sound pressure level). Noise exposure for either 1 or 3 hours induced significant elevations of threshold in auditory brainstem response (ABR) compared with unexposed controls. Succinate dehydrogenase staining showed that white-band noise exposure caused significant outer hair cell losses. In addition, we found significant up-regulations of cochlear Hes1 mRNA and protein expressions following acoustic trauma, and Hes1 mRNA expression was positively correlated with NIHL. These findings suggest that up-regulation of Hes1 expression in response to noise exposure may be one of the underlying mechanisms of NIHL.

Key words: acoustic trauma, hair cell, Hes1, noise-induced hearing loss

Living in a noisy environment has become a necessity due to increased mechanization in parallel with rapid industrialization. Consequently, noise has become a social and public health problem. Noise-induced hearing loss (NIHL) is one of the severest occupational diseases associated with excessive noise exposure (Nelson et al. 2005). Exposure to noise also has a significant effect on the cardiovascular system resulting in an increase in blood pressure. In addition, there is evidence that noise in the work environment might be a causal factor in work-related stress. Many anatomical, biochemical and physiological changes occur in the inner ear in response to overexposure to intense sound (Catlin 1986). However, the underlying mechanisms of NIHL have yet to be fully investigated.

As important inner ear sensory mechanoreceptors, auditory hair cells are vulnerable to damage from excessive noise exposure (Kopke et al. 1999). Although mammals (including rodents and humans) cannot spontaneously replace inner ear hair cells when lost, accumulating evidence suggests that the supporting cells on the organ of Corti can trans-differentiate into hair cells when appropriate signals are provided (Bermingham et al. 1999, Shou et al. 2003, Zheng and Gao 2000). Therefore, we assumed that providing positive or negative signals to promote cell differentiation in the inner ear might play a crucial role in treatment of noise-induced hair cell loss and hearing loss.

Specific genes control cell fate decision in the inner ear. At present, one of the pivotal genes for the control of inner ear hair cell differentiation appears to be the negative basic helix-loop-helix (bHLH) transcription factor, hairy and enhancer of split-1 (Hes1; Akazawa, 1992, Kageyama and Nakanishi 1997). Hes1 negatively regulates inner ear hair cell differentiation by antagonizing the action of the positive bHLH transcription factors such as Math1 (Zheng et al. 2000, Zine et al. 2001). Recently, Batts and coworkers (2009) reported that Hes1 protein expression was up-regulated in the aminoglycoside-damaged guinea pig organ of Corti, suggesting that the increase of Hes1 expression might be involved in the pathogenesis of ototoxic lesions

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(Batts et al. 2009). However, there is a lack of knowledge of relationship between Hes1 expression and NIHL in acoustic trauma.

In the present study, we showed a significant up-regulation of cochlear Hes1 mRNA and protein expressions from guinea pigs exposed to intense noise as compared with non-noise exposure controls. In addition, we found that the noise-induced up-regulation of Hes1 mRNA levels in the cochlea was positively correlated with the noise-induced hearing threshold elevation. Our results suggest that up-regulation of Hes1 level in response to noise exposure may be one of the mechanisms of NIHL.

Adult albino female guinea pigs (300–350 g) were kept at controlled room temperature under a 6:30 AM to 6:30 PM light regime. Food and water were available *ad libitum*. All studies were performed in accordance with the Guidelines for Animal Experiments of the Second Military Medical University, China.

The animals were exposed to white-band noise (Caravelli et al. 2004) at an overall level of 115 dB SPL (The frequency range is 0.2 -20 kHz) inside a sound exposure chamber with dimension of 1.2×1×0.8 m. Guinea pigs were exposed for 1 or 3 h. Noise signals were generated from a noise generator Brüel and Kjær 1405 (Kobenhavn, Denmark). The ambient sound level for "unexposed animals" was 30-40 dB SPL, and these animals served as controls.

Before and 24 hours after noise-exposure, auditory brainstem response (ABR) recordings were performed on both ears in both control and noise-exposed guinea pigs to evaluate hearing thresholds. Each animal underwent the ABR test after receiving an intraperitoneal pentobarbital sodium (40 mg/kg) injection. Active and reference needle electrodes were inserted subdermally in the vertex and ipsilateral retroauricular region, respectively, while a ground electrode was placed in the neck of the animal. Click stimuli (duration 0.1 ms) and tone burst (duration 5 ms, 1 ms rise/fall time) were delivered via a TDH-39P ear phone (Madsen Electronics, Kobenhavn, Denmark) to record the ABR (Spirit 2000, Nicolet USA), monaurally at a repetition rate of 11.1/s. The stimulus intensity began from 90-dB peak equivalent SPL, followed by 10-dB step decrements until waveforms I, III, and V disappeared, thus determining the ABR threshold.

After ABR recording, several animals were sacrificed for hair cell examination. The cochleas were quickly removed after deep anesthesia, and then perfused with a succinate dehydrogenase (SDH) staining solution (2.5 ml, 0.2 M sodium succinate, 2.5 ml, 0.2 M phosphate buffer pH 7.6, and 5 ml, 0.1% nitroblue tetrazolium) through the oval windows (Chen et al. 2000). The cochleas were then placed in the SDH solution and incubated at 37ºC for 55 minutes. The SDH-stained cochleas were post-fixed in 10% formaldehyde for more than 8 h at 4°C. The organ of Corti was dissected from the apex to the base, mounted in glycerin on microscope slides, coverslipped and examined using a phase contrast microscope (Zeiss, Jena, Germany) equipped with a digital camera (Zeiss).

For mRNA preparation, guinea pigs were decapitated immediately after ABR recording. The temporal bone was dissected and, using a dissecting microscope, the bony wall of the cochlea was removed in sterile PBS and the cochleas (two cochleas for each pool) were immersed in TRIzol reagent (GIBCO BRL Gaithersburg, MD) to extract total RNA according to the manufacturer's instructions. 2 μg RNA was reverse transcribed using superscript reverse transcriptase (Invitrogen) and stored at -20°C. The following sense and antisense primers were used: Hes1 (accession number NM_024360): 5'-CACCGGACAAACCAAAGACA -3' and 5'-AGCGGGTCACCTCGTTCA -3'. β-actin (accession number NM_031144): 5'-ATGGTGGGTATGGGTCAGAAGG -3' and 5'-TGGCTGGGGTGTTGAAGGTC -3'. Quantitative real-time PCR analysis was carried out in duplicates using Rotor Gene 3000 (Corbett Research, Sydney, Australia). Real-time PCR solution consisted of 40 ng diluted cDNA product, 0.1-0.3 μM of each paired primer, 2.5 mM Mg^{2+}, 100 μM deoxynucleotide triphosphates, 2 U Taq DNA polymerase, and 1× PCR buffer. SYBR green (BMA, Rockland, ME) was used as detection dye. Quantitative real-time PCR conditions were optimized according to preliminary experiments to achieve linear relationships between initial RNA concentration and PCR product. The annealing temperature was set at 60°C, and amplification cycles were set at 40 cycles. The temperature range to detect the melting temperature of the PCR product was set from 60–95°C. Amplification of the housekeeping genes β-actin for each sample as an internal control for sample loading and normalization. The melting curve was examined at the end of the amplification to ensure the specificity of PCR products. To determine the relative quantitation of gene expression for both target and housekeeping genes, the comparative Ct

(threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used (Livak and Schmittgen 2001).

To detect cochlear Hes1 protein expression, western blot analysis was carried out as described previously (Zhu et al. 2010). Briefly, guinea pigs were decapitated 24 hours after noise-exposure. The cochleas (two cochleas for each pool) were homogenized in cold T-Per lysis buffer (Pierce Biotechnology, Thermo Fischer, Rockford, IL). Then, lysates were quickly sonified in ice bath, boiled 5 min at 95°C, and stored at -80°C until used. Samples were separated on an SDS-10% polyacrylamide gel, and the proteins were electrophoretically transferred to a nitrocellulose filter at 300 mA for 90 min in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The filter was then blocked in TBS containing 0.1% Tween-20 (TBST) and 5% dried milk powder (wt/vol) for 2 h at room temperature. After three washes with TBST, the nitrocellulose filters were incubated with primary antibody against Hes 1 (1:1000 Chemicon, Temecula, CA, USA) at 4°C overnight. After another three washes with TBST, the filters were incubated with a secondary horseradish peroxidase-conjugated IgG (1:1000) for 1 h at room temperature and further washed for 30 min with TBST. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Santa Cruz). The light-emitting bands were detected with X-ray film. The resulting band intensities were quantitated using an image scanning densitometer (Furi Technology, Shanghai, China). To control sampling errors, the ratio of band intensities to β-actin was obtained to quantify the relative protein expression level.

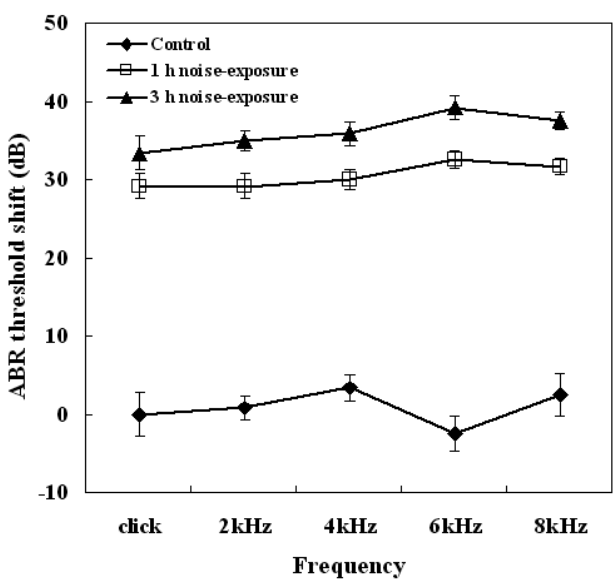

Fig. 1. ABR threshold shifts (mean ± SEM) of all animal groups tested (six animals/group). The guinea pigs were exposed to white-band noise at an overall level of 115 dB SPL for 1 h or 3 h. Unexposed animals served as controls. **$p<0.01$ *vs* control. #$p<0.05$ *vs* 1 h noise-exposure.

Means ± standard errors of the mean (SEM) were calculated of all parameters measured. The audiometric data were analyzed using two-way ANOVA. One-way ANOVA was used to detect statistically significant differences between the levels of ABR threshold shifts and Hes1 mRNA measured in guinea pig exposed to different amounts of noise. ANOVA was followed by the Student-Newmane-Keuls test to analyze individual means. Pearson's correlation was used for calculating the relations between ABR threshold shifts and Hes1 mRNA levels. A p-value<0.05 was considered statistically significant.

As shown in Fig. 1, the white-band noise exposure in this study induced significant elevations in the ABR threshold over the frequency ranges tested. The ABR threshold shift was 28 to 34 dB after 1 hour of noise exposure, and 33 to 37.5 dB after 3 hours of noise exposure. There were overall significant effects of noise exposure (both 1 hour and 3 hours) on the ABR threshold shift ($p<0.01$) compared to no-noise exposure controls, and the ABR threshold shift was significantly higher after 3 hours of noise exposure than after 1 hour of noise exposure ($p<0.05$). In the control animals, auditory thresholds did not change as compared to their initial values. We then used one-way ANOVA to detect the statistical significant differences between the levels of ABR threshold shifts in various frequencies and auditory clicks. It was found that 1 and 3 hours of noise exposures resulted in significant ABR threshold shifts at the same level ($p<0.01$) in all frequencies including auditory clicks compared to no-noise exposure controls. In addition, the ABR threshold shifts caused by 3 hours of noise exposure were significantly higher than those caused by 1 hour of noise exposure in all tested frequencies ($p<0.05$), but not in auditory clicks.

Cochlear hair cells have much higher SDH activity than other cells of the organ of Corti. Thus, they are clearly recognized in the surface preparation stained by SDH solution (Chen et al. 2000). Fig. 2 shows examples of SDH-stained hair cells from the middle turns (50% from the apex) of noise-exposed guinea pigs. In control

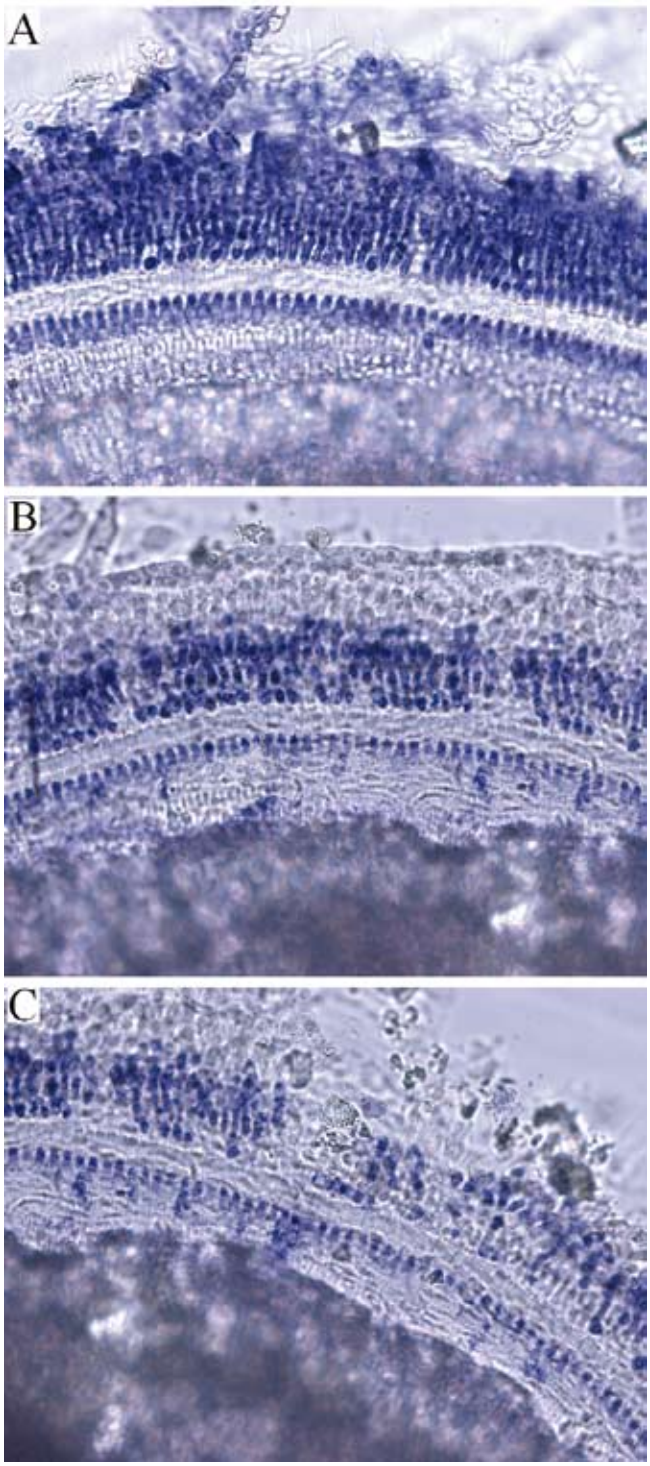

Fig. 2. Examples of SDH-stained hair cells in subjects from the middle turns (50% from the apex). A, unexposed guinea pigs. B and C, animals exposed to white-band noise for 1 h and 3 h, respectively. Red arrows point to OHCs and white arrows point to IHCs. White arrowheads point to the missing hair cells. Horizontal bars represent 50 μm length.

animals, a normal geometric array without hair cell loss was observed, with one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs, Fig. 2A). As shown in Fig. 2B and C, the white-band noise used in the present study caused significant OHCs losses in noise-exposed animals compared to no-noise exposure controls. The OHCs losses were much more severe after 3 hours of noise exposure than after 1 hour of noise exposure. In addition, the IHCs did not show any apparent damage.

Using a real-time quantitative PCR approach, the changes of Hes1 mRNA expression in the cochleas were measured after white-band noise exposure. To ensure that total RNA levels were comparable in each sample, the abundance of Hes1 mRNA expression was normalized to the housekeeping gene β-actin. As shown in Fig. 3A, clear up-regulations of Hes1 mRNA expression in the cochlea by 1.5- and 2.5- fold occurred after white-band noise exposure for 1 and 3 hours, respectively. However, as for Hes1 protein expression level, it was found that only longer period of noise exposure (3 hours) resulted in significant elevation of cochlear Hes1 protein expression compared with control animals. The Hes1 mRNA and protein levels were both significantly higher after 3 hours of noise exposure than after 1 hour of noise exposure ($p<0.01$). In addition, we found that the white-band noise-induced up-regulation of Hes1 mRNA levels in the cochlea was positively correlated with the mean threshold elevation (Fig. 3B).

The literature relating NIHL to inner ear hair cell loss is extensive, but the findings have been contradictory (Chen and Fechter 2003). For instance, Hamernik and coauthors (1989) reported consistent OHC losses with less than 5-dB permanent hearing threshold shifts in chinchillas. On the other hand, Borg (1987) demonstrated that hair cell loss was not found until NIHL exceeded about 30 dB in normotensive albino rats. In the present study, we found that 1 hour of white-band noise exposure caused a NIHL of 28 to 32 dB, and 3 hours of noise exposure caused a NIHL of 33 to 37.5 dB. In addition, although the NIHL induced by 3 hours of noise exposure was only about 5 dB larger than that induced by 1 hour of noise exposure, the related OHC losses were much more severe in the 3-hour noise exposure group than in the 1-hour noise exposure group. Previous reports have demonstrated that NIHL may be due to pathologic bases such as stria vascular degeneration, and supporting cell injury and neural

degeneration other than hair cell losses (Le Prell et al. 2007). We assume that the complicated cochlear pathology may account for the disparate effects of noise exposure on hearing loss and hair cell loss.

Consistent with reports in the literature (Loquet et al. 1999, Pouyatos et al. 2002), our results indicate that the noise-induced loss of OHCs is quite greater than that of the IHCs. The greater vulnerability of OHCs to noise injury is generally considered to be due to their electromotility and increased metabolic needs, hence the generation of free radicals that cause cell death by apoptosis or necrosis (Henderson et al. 2006).

The IHCs and OHCs are derived from the greater epithelial ridge (GER) and the lesser epithelial ridge (LER) cells, respectively, during embryogenesis (Lim and Rueda 1992). As one of the most crucial genes for the control of hair cell differentiation, Hes1 has been found to be expressed selectively in GER and LER regions which are adjacent to IHCs and OHCs, respectively, in the cochlea (Zhang et al. 2007, Zine et al. 2001). When the Hes1 gene was deficient, there were supernumerary hair cells in the cochlea (Zheng et al. 2000). These findings confirm that Hes1 plays important roles in hair cell differentiation; however the status of the Hes1 gene in the damaged mammalian auditory epithelium has not been well characterized. Recently, Batts and others (2009) reported that Hes1 protein expression was up-regulated following an ototoxic lesion. In the present study, we found that Hes1 expression was significantly up-regulated following a noise-induced lesion. In addition, we found that the noise-induced up-regulation of Hes1 mRNA level in the cochlea was closely related to NIHL, suggesting that the relationship between acoustic trauma and the Hes1 level might be one of the mechanisms of NIHL.

Hes1 is a candidate target gene of the Notch signaling pathway (Ohtsuka et al. 1999). It has been reported that the constitutively active form of Notch (caNotch) can activate both Hes1 promoter activity and endogenous Hes1 expression (Jarriault et al. 1995, Nishimura et al. 1998). In addition, the exogenous Notch ligand, Delta, can also induce Hes1 expression (Jarriault et al. 1998, Wang et al. 1998). Batts and colleagues (2009) recently reported that a severe ototoxic lesion to the organ of Corti not only increased Hes1 expression but also up-regulated Notch signaling. Therefore, they assumed that mammalian supporting-cell trans-differentiation might be repressed by Notch-dependent Hes activity after hair cell loss. According to our findings, a similar mechanism may also account for noise-induced hair cell loss. The status of Notch/Notch ligands in the cochlea following noise exposure needs further investigation.

In summary, the present study showed that white-band noise exposure for either 1 or 3 hours induced significant NIHL and OHCs losses. In addition, we found a significant up-regulation of cochlear Hes1 expression following acoustic trauma, which was

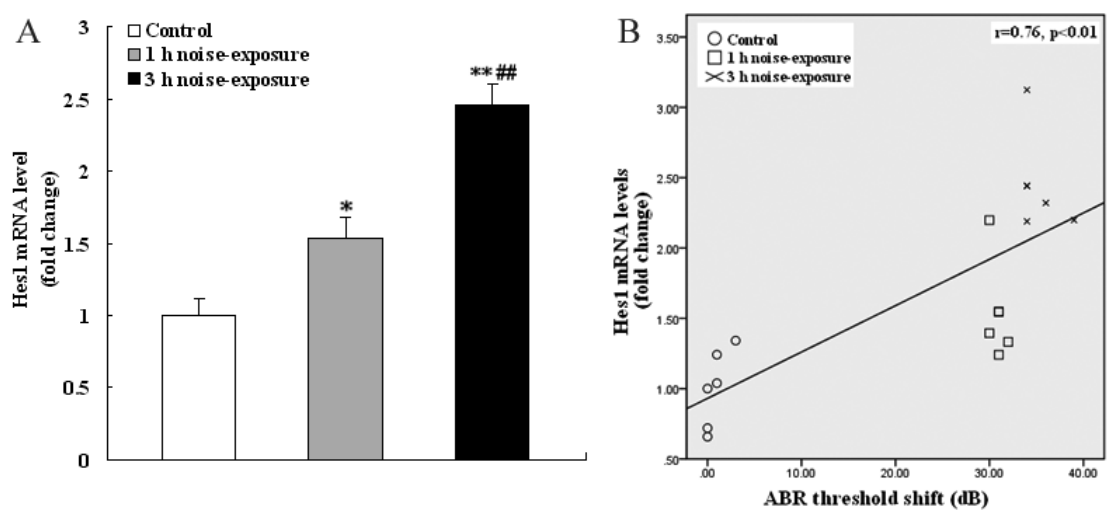

Fig. 3. Cochlear Hes1 expression in response to noise exposure. A. The guinea pigs were exposed to white-band noise for 1 h or 3 h. Unexposed animals served as controls. Quantitative real-time RT-PCR and Western blot were used to determine mRNA and protein expression of Hes1, respectively, in cochleas. Data were expressed as mean fold changes of control ± SEM (n=6). *$p<0.05$, **$p<0.01$ *vs* control, ##$p<0.01$ *vs* 1 h noise-exposure. B. Correlation between cochlear Hes1 mRNA expression and the mean ABR threshold shifts of all animals tested.

positively correlated to the NIHL. These findings suggest that the relationship between acoustic trauma and Hes1 level may be one of the mechanisms of NIHL. In addition, previous investigation has showed that loss of Hes1 gene will lead to increased numbers of hair cells in the mammalian inner ear (Zine et al. 2001), suggesting that genetic or pharmacological inhibition of Hes1 expression might have significant therapeutic value in treatment of NIHL.

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