Role of cysteinyl leukotriene signaling in a mouse model of noise-induced cochlear injury

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Noise-induced hearing loss is one of the most common types of sensorineural hearing loss. In this study, we examined the expression and localization of leukotriene receptors and their respective changes in the cochlea after hazardous noise exposure. We found that the expression of cysteinyl leukotriene type 1 receptor (CysLTR1) was increased until 3 d after noise exposure and enhanced CysLTR1 expression was mainly observed in the spiral ligament and the organ of Corti. Expression of 5-lipoxygenase was increased similar to that of CysLTR1, and there was an accompanying elevation of CysLT concentration. Posttreatment with leukotriene receptor antagonist (LTRA), montelukast, for 4 consecutive days after noise exposure significantly decreased the permanent threshold shift and also reduced the hair cell death in the cochlea. Using RNA-sequencing, we found that the expression of matrix metalloproteinase-3 (MMP-3) was up-regulated after noise exposure, and it was significantly inhibited by montelukast. Posttreatment with a MMP-3 inhibitor also protected the hair cells and reduced the permanent threshold shift. These findings suggest that acoustic injury up-regulated CysLT signaling in the cochlea and cochlear injury could be attenuated by LTRA through regulation of MMP-3 expression. This study provides mechanistic insights into the role of CysLTs signaling in noise-induced hearing loss and the therapeutic benefit of LTRA.

The number of people with hearing impairment is rapidly increasing, and it has become an important health care issue. Excessive noise exposure is one of the most common causes of sensorineural hearing loss. Noise-induced hearing loss (NIHL) is one of the most common occupational diseases, and it has also been proposed as a contributor to presbycusis (1). Moreover, there are some concerns regarding the negligent use of portable listening devices or noise exposure in leisure environments such as nightclubs or music concerts as potent causes of NIHL (2, 3). In these populations, wearing hearing protection devices, the current most effective method for preventing NIHL, was not useful, thereby indicating that additional strategies are needed (4).

Great efforts have been made to elucidate the pathogenesis of NIHL. Accumulating evidence indicates that noise induces an intense metabolic activity, which is the primary cause of reactive oxygen species (ROS) overproduction, and overproduced ROS have been known to be the major contributors to NIHL. In addition, several pathophysiological mechanisms including oxidative stress, excitotoxicity, ischemia/reperfusion injury in the stria vascularis, ion imbalance in the endolymph, and inflammatory responses have been proposed to be involved in the pathogenesis of NIHL (5–7).

Leukotrienes (LTs) are potent lipid mediators in response to several immune and inflammatory stimuli. In particular, cysteinyl LTs (CysLTs), including LTC4, LTD4, and LTE4, have been known to be involved in several inflammatory responses (8). The actions of CysLTs are mediated via at least two types of CysLT receptors, designated as CysLT receptor 1 (CysLTR1) and 2 (CysLTR2). CysLTs–CysLTR1 signal has been considered a major pharmacological target for the treatment of asthma and allergic rhinitis (9). Recently, expanded roles of this signaling in the pathogenesis of cardiovascular diseases, cerebrovascular diseases, malignant tumors, fibrosis, and immune host defense (10, 11) have been suggested, thereby indicating that they could be involved in a variety of pathophysiological conditions as well as in inflammation in a tissue-specific manner.

To elucidate the involvement of LT signaling in the auditory system in association with excessive noise exposure, we explored the detailed expression and regulation of LTs and their receptors in a mouse model of noise-induced cochlear injury and the protective effect of LTRA on NIHL.

Results

LT Receptor Gene Expression in the Normal and Noise-Exposed Cochlear Tissues of Mice. First, we performed mRNA expression analysis of CysLT receptors and Leukotriene B4 (LTB4) receptors in the normal cochlear tissue of mice. The endogenous mRNAs of CysLTR1 (*Cysltr1*), CysLTR2 (*Cysltr2*), and BLT1 (*Ltb4r1*) were expressed in normal cochleae. In contrast, the mRNA of BLT2 (*Ltb4r2*) was hardly detected (Fig. 1*A*). To assess the role of LT receptors in noise-induced cochlear injury, we examined the temporal transcriptional expression patterns of LT receptors after hazardous noise exposure. Mice were exposed to broadband white noise at 112 dB sound pressure level (SPL) for 3 h. Then, cochleae were obtained at 0 (control), 1, 3, 7, and 14 d after noise exposure. We found that *Cysltr1* expression increased gradually and it was significantly increased until 3 d and declined to the basal expression level at 7 d after noise exposure (Fig. 1*B*). In contrast, excessive noise did not have a significant effect on the expression of *Cysltr2*, *Ltb4r1*, and *Ltb4r2* (Fig. 1 *C*–*E*). Expression of the LT synthetic enzyme arachidonic acid 5-lipoxygenase mRNA (*Alox5*) was also increased

Significance

Cysteinyl leukotriene signaling has been known to play an important role in inflammation, and its inhibition has been commonly performed to treat asthma and allergic rhinitis. In this study, we demonstrated that cysteinyl leukotriene signaling is involved in the pathogenesis of noise-induced cochlear injury and its inhibition by montelukast attenuated noise-induced hair cell death and hearing loss. In addition, we showed that the effects of montelukast on noise-induced hair cell death are mediated through the regulation of matrix metalloproteinase-3 expression. Accordingly, this study will be helpful for developing new therapeutic strategies for noise-induced hearing loss, and it will serve as a basis for investigating other hearing loss disorders in which leukotriene signaling is involved.

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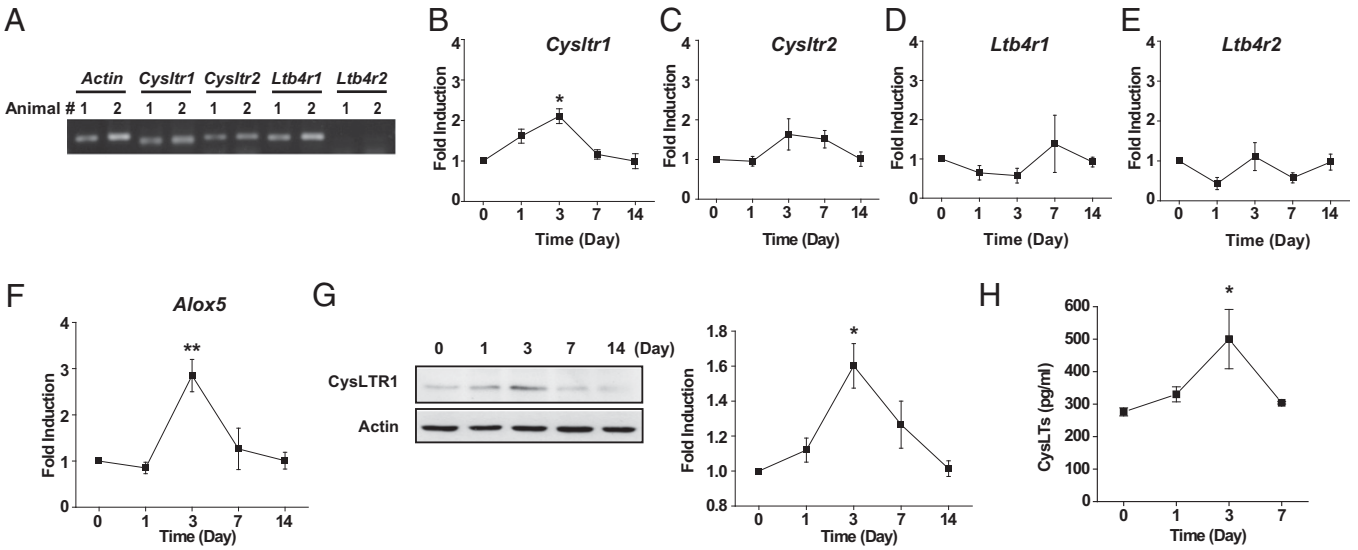

Fig. 1. The mRNA expression of LT signaling components in the control and noise-exposed cochleae. (*A*) The mRNA expression of LT receptors in the normal cochlea from two animals. (*B–F*) Quantitative real-time PCR results of *Cysltr1*, *Cysltr2*, *Ltb4r1*, *Ltb4r2*, and *Alox5* in the noise-exposed cochleae obtained at 0 (control), 1, 3, and 7 d after noise injury. Presented data were obtained using eight cochleae from four animals per time point. (*G*) Western blot analysis results of CysLTR1 in the noise-exposed cochleae after noise injury (eight cochleae from four animals per time point). Presented data were obtained from four independent experiments. (*H*) Concentration of CysLTs in the cochlear homogenates of four groups was determined by enzyme immunoassay. Six cochleae from three animals per time point were analyzed. Results are shown as means ± SEM. $*P < 0.05$, $**P < 0.01$ compared with control.

after noise exposure, and synchronization of the mRNA pattern with *Cysltr1* was observed (Fig. 1*F*). The expression of CysLTR1 protein showed a similar pattern to that of *Cysltr1* (Fig. 1*G*). Next, we examined the concentrations of CysLTs in cochlear homogenates. The concentrations of CysLTs started to increase at 1 d and peaked at 3 d after noise exposure, and then, they returned to the basal level at 7 d after noise exposure (Fig. 1*H*).

Temporal and Spatial Expression Patterns of CysLTR1 After Noise Exposure. We continued to investigate the spatial expression pattern of CysLTR1 by immunohistochemical analysis. It is known that the basal turn area is the most vulnerable site for acoustic overstimulation (4). Therefore, we focused on the changes in expression in the basal turn area. Before noise exposure, the expression of CysLTR1 was localized to the organ of Corti (OC), and the expression level was low (Fig. 2 *A–C*). Neither the spiral ligament nor the stria vascularis of the normal cochlea contained CysLTR1-positive cells. However, Fig. 2 *D–F* shows that CysLTR1 expression was slightly increased in the spiral ligament and stria vascularis at 1 d after noise exposure. The increase of CysLTR1 expression peaked in the OC, stria vascularis, and spiral ligament at 3 d after noise exposure (Fig. 2 *G–I*). CysLTR1 labeling was minimal at 7 d compared with the peak level (Fig. 2 *J–L*). Collectively, these results imply that the CysLTs–CysLTR1 signaling pathway may contribute to the development of the subacute phase rather than to the development of the acute or chronic phase of noise-induced cochlear injury.

Blockade of CysLTR1 Signaling by Montelukast Attenuated Noise-Induced Cochlear Injury. To evaluate the involvement of the CysLTR1 signaling pathway in NIHL, we used montelukast, a specific CysLTR1 antagonist, to inhibit this signaling. We treated the mice with montelukast for 4 d after noise exposure. Then, the auditory brainstem response (ABR) analysis was performed. In the compound threshold shift (CTS) analysis performed at 1 d after noise exposure, there was no difference between the noise-only group and the noise-montelukast group. However, in the permanent threshold shift (PTS) analysis performed at 14 d after noise exposure, PTS was markedly reduced in the noise-montelukast group compared with the noise-only group (Fig. 3 *A* and *B*). Next, to evaluate whether montelukast has a protective effect on hair cell death after noise injury, we first performed the surface preparation analysis in the basal turn area at 14 d after noise exposure, revealing that the survival rate of outer hair cells (OHCs) in the noise-only group was significantly decreased. However, montelukast treatment significantly reversed the survival rate of OHCs (Fig. 3 *C* and *D*). Second, scanning electron microscopy (SEM) images also showed that the damage to the stereocilia on OHCs was decreased in the noise-montelukast group compared with the noise-only group (Fig. 3*E*), suggesting that blockade of CysLTR1 signaling reduced noise-induced hair cell injury significantly. Montelukast treatment without noise exposure did not affect the baseline ABR or hair cell survival (Fig. S1 *A–C*). In addition, we examined the changes in expression of *Cysltr1* to address the question of whether montelukast treatment could also affect the induction of CysLTR1 expression. Montelukast effectively reduced the upregulation of *Cysltr1* expression after noise exposure (Fig. 3*F*), suggesting that noise-induced changes in gene expression were also affected by the inhibition of CysLTR1 signaling.

Neither ROS Generation Nor RNS Generation Was Decreased by Montelukast. The overproduced ROS and reactive nitrogen species (RNS) after excessive noise exposure have been known to be the major contributors to NIHL (4, 12–14). Furthermore, the inhibition of CysLT signaling has been reported to have an antioxidant effect on vascular endothelial injury, myocardial injury, and gentamicin-induced renal injury (15–17). Thus, we first examined whether montelukast could reduce the formation of ROS and RNS in the cochleae injured by noise exposure. 4-Hydroxynonenal (4-HNE) is a product of membrane peroxidation that is generated by the reaction of free radicals in the plasma membrane and is known to be produced in cochleae damaged by sound-induced trauma (13, 18). To evaluate whether montelukast treatment attenuates ROS generation after acoustic overstimulation, serial immunohistochemistry for 4-HNE was performed. After noise exposure, 4-HNE expression was strongly detected in the stria vascularis, spiral ligament, and OC in the

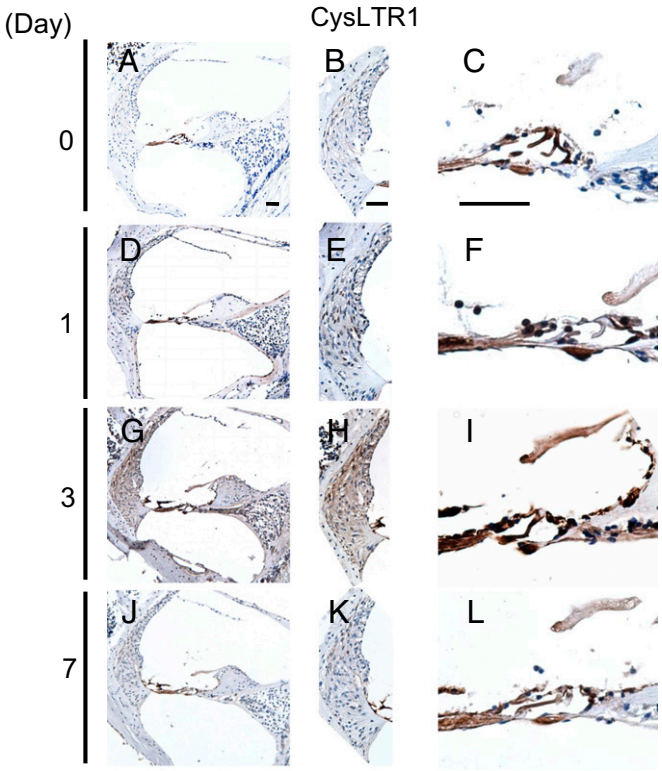

Fig. 2. Immunohistochemical analysis of the spatial and temporal expression of CysLTR1 in the cochleae after acoustic overstimulation. After acoustic overstimulation, paraffin-embedded sections were incubated with anti-CysLTR1 antibody, followed by 3, 3′-diaminobenzidine (DAB) staining. Data shown are representative of three independent experiments. (*A–C*) Control group, (*B–F*) postexposure day 1 group, (*G–I*) postexposure day 3 group, and (*J–L*) postexposure day 7 group. Higher magnification images (*B*, *E*, *H*, *K* and *C*, *F*, *I*, *L*) were focused on the lateral wall of the cochlea and the OC, respectively. (Scale bar, 50 μm.)

noise-only group compared with the control group. In the noise-montelukast group, 4-HNE expression was not significantly decreased compared with that in the noise-only group (Fig. 4*A*). 3-Nitrotyrosine (3-NT) can be used as a marker for oxidation and nitration products, which reflect the reaction with peroxynitrite (ONOO-) (19). Serial immunohistochemistry for 3-NT showed that it was strongly detected in the lateral wall and OC in the noise-only group compared with the control group. 3-NT expression was also not attenuated in the noise-montelukast group compared with the noise-only group (Fig. 4*B*), which was similar to the result for 4-HNE. These results suggest that inhibition of CysLTR1 signaling did not alter the generation of ROS and RNS in the cochlea after acoustic overstimulation.

RNA-Sequencing Data Revealed Novel Gene Expression Patterns in the Noise-Exposed Cochlea. To investigate the possible linkage between montelukast treatment and hair cell protection from excessive noise, we performed RNA-sequencing (RNA-seq) to obtain comprehensive mRNA expression profiles at 3 d after noise exposure. After data processing, we obtained an average of 24 M reads per sample, which were mapped to the reference mouse genome (Table S1). First, we identified 146 differentially expressed genes (DEGs) with a greater than twofold difference between the noise-treated and control groups. When we performed the gene set enrichment analysis using the DEGs, inflammation-related genes were found to be enriched in the noise-only group compared with the control group (Table S2). On the contrary, the inflammation and chemotaxis-regulated gene functions were significantly down-regulated by the treatment with montelukast compared with that in the noise-only group. This may suggest that the inflammation-related genes play pivotal roles in noise injury and montelukast has a protective effect against noise injury. The genes up-regulated by posttreatment with montelukast did not show a statistical significant functional enrichment. Next, to pinpoint the responsible genes for the noise injury and/or montelukast-treatment effects, we identified the 12 genes by applying four group ANOVA tests with stringent criteria of $P < 0.005$ and fold differences greater than 2 in the noise-treated groups but fold differences less than 2 in the other groups compared with control group. Among these, nine genes including solute carrier family 13, member 2 (*Slc13a2*), *Adam28*, regenerating islet-derived 3 gamma (*Reg3g*), *matrix metalloproteinase-3* (*Mmp3*), *Ighv1–73*, pulmonary surfactant-associated protein D (*Sftpd*), VEGF coregulated chemokine 1 (*Cxcl17*), tumor-associated calcium signal transducer 2 (*Tacstd2*), and general transcription factor IIE, subunit 2 (*Gtf2e2*) were up-regulated by noise exposure, and their expressions were reversed by posttreatment with montelukast (Fig. 5). This result suggests that these nine genes might be putative targets for the effect of montelukast on NIHL.

Inhibition of MMP-3 After Noise Exposure Attenuated Cochlear Injury. Of the nine putative target genes responsible for the effect of montelukast, *Mmp3* was of particular interest, because *Mmp3* was previously shown to be up-regulated in the noise-exposed cochlea and excessive activity of MMP-3 was found to be associated with cell death (20, 21). Therefore, we further analyzed the expression patterns of a series of MMP genes and their endogenous inhibitors, tissue inhibitors of metalloproteinase genes from the expression data. Notably, *Mmp3* showed the most prominent change in expression after noise exposure, and this change in the expression of *Mmp3* was reversed by montelukast treatment (Fig. S2). This finding could be validated by the quantitative PCR experiment. Posttreatment with montelukast effectively inhibited the increase in *Mmp3* expression at 3 d after noise exposure (Fig. 6*A*). We also examined the levels of MMP-3 protein in mouse cochlear homogenates by Western blot. Indeed, the elevation of MMP-3 expression after noise injury was significantly reversed by montelukast treatment (Fig. 6*B*). In addition, we investigated the spatial expression pattern of MMP-3 protein in mouse cochlear sections by immunohistochemistry (Fig. 6*C*). Sections of the control and the montelukast groups with or without noise exposure showed very little MMP-3 immunoreactivity. However, the noise-only group showed up-regulation of MMP-3 mainly in the spiral ligament (Fig. 6*D*). To determine whether montelukast treatment could protect the cochlea from acoustic overstimulation by inhibiting MMP-3 expression, we measured PTS at 14 d after excessive noise exposure with or without treatment by MMP-3 inhibitor (Fig. 6*E*), indicating that treatment with MMP-3 inhibitor significantly decreased the threshold shift compared with that in the noise-only group. Hair cell morphology assay using SEM also confirmed that MMP-3 inhibition after noise exposure alleviated cochlear injury, thereby suggesting that the inhibition of CysLTR1 signaling by montelukast attenuated cochlear injury by inhibiting MMP-3 expression. The MMP-3 inhibitor treatment with no noise exposure did not affect the baseline ABR or hair cell survival (Fig. S1 *D–F*).

Discussion

In the present study, we investigated the novel role of CysLT signaling in a mouse model of noise-induced cochlear injury. Although there are a few studies assessing the involvement of eicosanoids in various animal models of hearing loss (22–24), to our knowledge, this is the first reported study to show the involvement of CysLTR1 signaling in the pathogenesis of noise-induced cochlear injury in mice. This study shows that CysLTR1

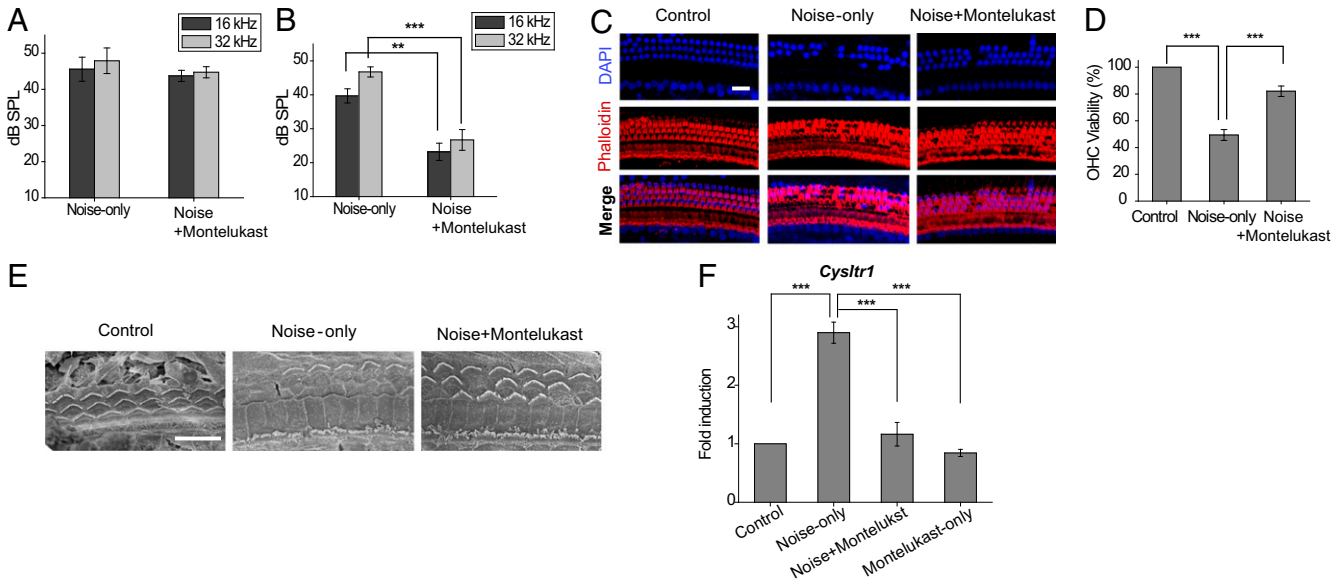

Fig. 3. Otoprotective effect of posttreatment with montelukast. (*A* and *B*) ABR threshold shift at 16 kHz and 32 kHz was measured at day 1 (CTS) (*A*) and at day 14 (PTS) (*B*) after noise exposure (noise-only group, 12 animals; montelukast group, 16 animals). (*C*) After acoustic overstimulation, surface preparation was performed. Then, hair cells were stained with DAPI (blue) and Texas Red VR-X phalloidin (red) and observed under a confocal microscope. (Scale bar, 20 μm.) (*D*) Hair cell loss in the cochlear basal turn was measured using a cytocochleogram at day 14. The *P* value was applied to ANOVA with Tukey (three cochleae from three animals in the control, six cochleae from six animals in the noise-only group, and 10 cochleae from 10 animals in the montelukast group). (*E*) After acoustic overstimulation, SEM analysis was performed. Data shown are representative of three independent experiments. Twelve cochleae from six animals per group were examined at day 14. (Scale bar, 10 μm.) (*F*) Quantitative real-time PCR result of *Cysltr1* in the control group, noise-only group, noise + montelukast group, and montelukast-only group at 3 d after noise injury. Presented data were obtained using 12 cochleae from six animals per group. $^{**}P < 0.01$, $^{***}P < 0.001$.

and CysLTR2 were expressed and CysLTs were synthesized in the normal cochlea, suggesting that CysLTs–CysLTRs signaling may play a physiological role in maintaining cochlear homeostasis. Moreover, excessive noise exposure increased the expression of CysLTR1 and 5-LO and further increased the level of CysLTs in the subacute phase of cochlear injury, indicating that CysLTs–CysLTR1 signaling may have a role in the pathogenesis of NIHL. Indeed, the blockade of CysLTR1 signaling by montelukast attenuated NIHL, even though we posttreated the mice with montelukast after noise exposure.

CysLTR1 antagonists such as montelukast and pranlukast have been reported to protect against ischemia/reperfusion injury in a variety of tissues such as in the heart, liver, kidneys, and spinal cord, and the mechanism of these effects was suspected to involve a decrease in inflammation and oxidative stress (25–29). As inflammation and oxidative stress are also known to contribute to NIHL, we first explored whether montelukast protected against noise-induced cochlear injury through the regulation of oxidative stress. In our model system, montelukast treatment did not attenuate the production of ROS or RNS induced by excessive noise, suggesting that the protective effect of montelukast was not mediated via a decrease in oxidative stress. Instead, it may be mediated via the downstream level of ROS overproduction or the ROS-independent signaling pathways. Our gene set enrichment analysis revealed that the inflammation-related genes played a pivotal role in the pathogenesis of NIHL and montelukast had effects in regulating them. However, in our experimental condition, an increase in proinflammatory cytokines such as TNF-α and IL-1β in the cochlear tissue after excessive noise exposure was not observed (Fig. S3*A*). Although phagocyte infiltration was observed in the cochlea at 7 d after excessive noise exposure and it was decreased by montelukast treatment (Fig. S3*B*), phagocyte infiltration occurs in the late phase of noise-induced cochlear injury, and it has been considered to play a role in the removal or reconstitution of the damaged structures rather than in the augmentation of the cochlear injury (6). Accordingly, the protective effects of montelukast in the cochlea were unlikely to be due to its direct anti-inflammatory effect, although we could not completely exclude the possibility of an anti-inflammatory role of montelukast in NIHL.

Analysis of RNA-seq data demonstrated that nine genes, including metalloproteinases such as *Mmp3* and *Adam28*, were up-regulated after noise exposure and the expression changes were reversed by montelukast. *Cxcl17*, *Reg3g*, and *Sftpd* may be associated with the inflammatory response. *Slc13A2* is a gene encoding a sodium-dependent dicarboxylate transporter that may play a role in oxidative metabolism in the cochlea (30). However, other genes, including *Tacstd2* and *Gtf2e2*, have not been studied in the context of cochlear injury. Thus, further work is required to identify the exact roles of these genes in the subacute phase of noise injury. In this study, we focused on MMP-3 expression. Interestingly, immunohistochemical analysis revealed

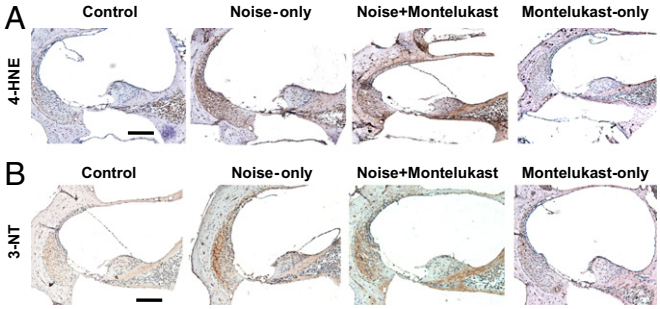

Fig. 4. Immunohistochemical analysis of 4-HNE and 3-NT after acoustic overstimulation. After acoustic overstimulation, paraffin-embedded sections were incubated with anti–4-HNE antibody (*A*) and anti–3-NT antibody (*B*), followed by DAB staining. Data shown are representative of three independent experiments (three cochleae from three animals each at day 7). (Scale bar, 100 μm.)

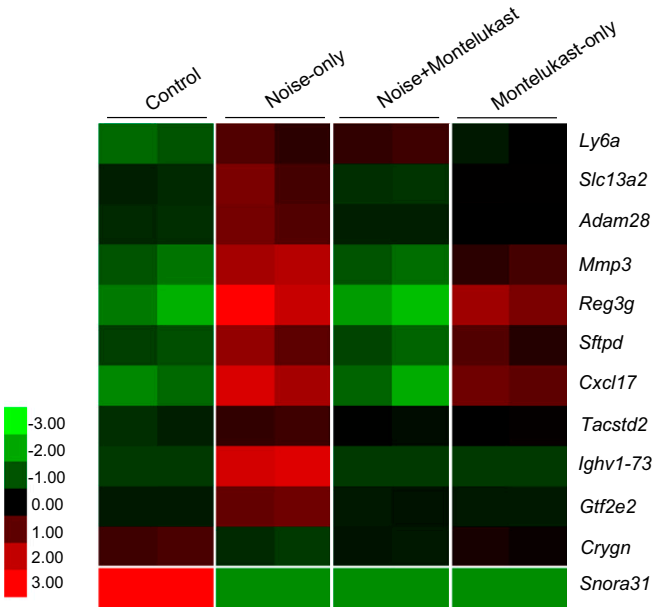

Fig. 5. DEGs among the groups. The heatmap shows 12 DEGs that were identified by ANOVA test among the four groups ($P < 0.005$). The expression levels indicate the log2-transformed and gene-centered values across the samples.

that MMP-3 was specifically up-regulated in the spiral ligament, where CysLTR1 was also up-regulated after noise exposure. Given that fibrocytes in the spiral ligament play an important role in maintaining cochlear homeostasis (31) and the spiral ligament expresses extracellular matrix proteins such as fibronectin, laminin, collagen IV, and proteoglycans, which are the substrates of MMP-3 (32–34), disruption of spiral ligaments by excessive MMP-3 activity could induce cochlear injury, and it could be a target for the preventive effect of montelukast. Here, we demonstrated that treatment with an MMP-3 inhibitor reduced hair cell death as well as PTS. Consistent with our data, it has been recently reported that MMPs contribute to noise-induced cochlear injury, although they observed that inhibition of MMP activity with doxycycline reduced or potentiated the PTS and hair cell loss in a time-dependent manner (20), and this result might be due to the overall inhibition of all MMP activities by doxycycline.

The reduced level of threshold shift by an MMP-3 inhibitor was slightly less than that by montelukast, implying that it is highly likely that CysLTR1 signaling may also function through the regulation of other apoptotic pathways. Eicosanoids including prostaglandins and LTs have been known to contribute to the vascular phase of inflammation and to act differently according to their end products (10). Accumulating evidence indicates that CysLTs function as arterial vasoconstrictors. In contrast, prostaglandin E2 (PGE2), the most abundant cyclooxygenase derivative, is a well-known vasodilator (35). Disruption of cochlear blood flow has been suggested to induce hypoxia/reperfusion injury in the stria vascularis, leading to hair cell death (36). It has been reported that administration of salicylate or nonsteroidal anti-inflammatory drugs causes mild-to-moderate reversible hearing loss and tinnitus, and these changes are associated with decreased levels of PGs and increased levels of LTs in perilymphatic fluids (37), postulating the association between ototoxicity and vascular structural changes induced by eicosanoids. Reduced circulation in the cochlea in response to noise exposure has been reported, and alteration of cochlear blood flow has been known to play a role in the pathogenesis of NIHL (1, 5). Accordingly, based on the previous studies and our present data, which demonstrate that excessive noise exposure increased the

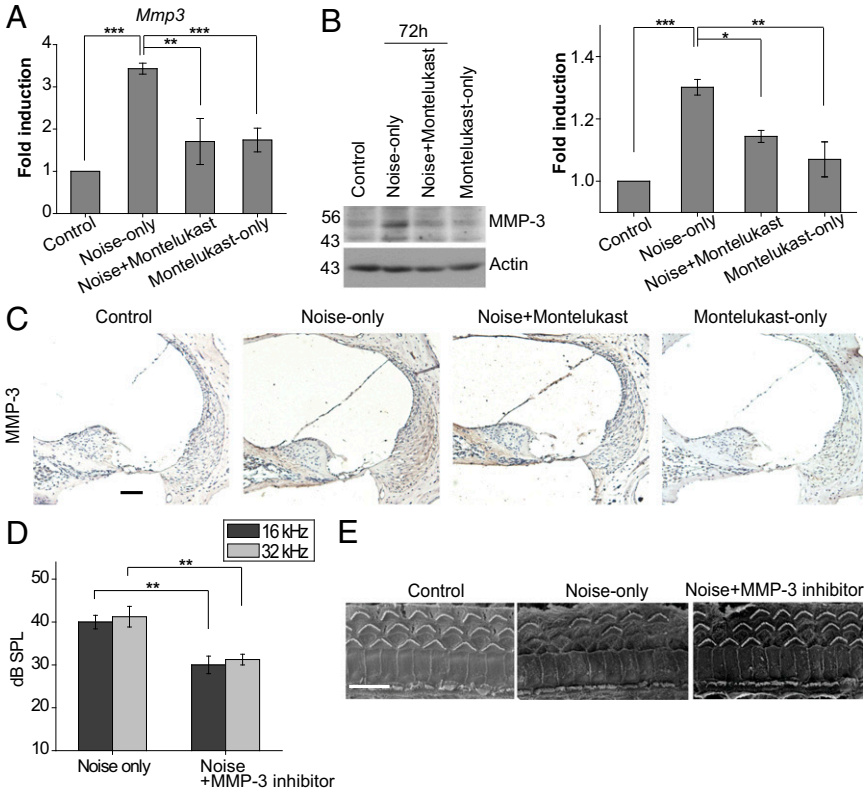

Fig. 6. Involvement of MMP-3 in the pathogenesis of CysLT signaling-related noise-induced cochlear injury. (*A*) Quantitative real-time PCR result of *Mmp3* obtained at 3 d after noise exposure. Presented data were obtained from four independent experiments (16 cochleae from eight animals per group). (*B*) Western blotting analysis results of MMP-3 obtained at 3 d after noise exposure. Data shown are representative of three independent experiments (six cochleae from three animals per group). Presented data were analyzed using densitometric analysis. The intensities of the actin bands were used for normalization. (*C*) After acoustic overstimulation, paraffin-embedded sections were incubated with anti–MMP-3 antibody followed by DAB staining. Data shown are representative of three independent experiments (three cochleae from three animals each at day 3). (Scale bar, 50 μm.) (*D*) ABR threshold shift at 16 kHz and 32 kHz was measured at day 14 (PTS) after noise exposure (noise-only group, four animals; noise + MMP-3 inhibitor group, eight animals). (*E*) After acoustic overstimulation, SEM was also performed. Data shown are representative of three independent experiments. Six cochleae from three animals per group were examined at day 14. (Scale bar, 10 μm.) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

level of CysLTs in the cochlear tissue and the expression of CysLTR1 in the lateral wall of the cochlea, the increased level of CysLTs may cause vasoconstriction in the cochlear tissue, which could contribute to cochlear injury, and administration of montelukast inhibits the vasoconstrictor effect of CysLTs, which may protect against NIHL. Furthermore, recent evidence suggests that montelukast possesses a range of secondary activities independent of CysLTR1, including inhibition of 5-LO, histone acetyltransferase, cAMP phosphodiesterase, and interference with purinergic P2Y receptors (38–40). In particular, P2Y receptors have been reported to be linked to NIHL; P2Y receptors are widely expressed in the cochlear tissue and play a role in the maintenance of K^+ transport and cycling (41). In addition, acoustic overstimulation induces ATP release (42) and upregulates the expression of P2Y6 and P2Y14 receptors in the cochlea (41). P2Y receptors have also been known to be involved in the propagation and sensing of noise damage (43). Although posttreatment with the P2Y6 receptor antagonist MRS2578 after noise exposure showed neither a protective nor a synergistic effect with montelukast on NIHL (Fig. S4), an effect of montelukast independent of CysLTR1, including through other purinergic receptors, could also attenuate NIHL. In summary, we observed that CysLTs and their receptors were expressed in the normal mice cochlea, and noise injury to the cochlea was associated with the activation of CysLTR1-mediated signaling. Antagonizing the CysLTR1 signaling by montelukast after noise injury significantly attenuated noise-induced threshold shift and hair cell injury, and this implies the possibility of its use as an effective posttreatment agent. Therefore, this study will be helpful for developing new therapeutic strategies for NIHL, and it will serve as a basis for investigating other hearing loss disorders in which LT signaling is involved.

Materials and Methods

All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee at Ajou University. Acoustic overexposure was performed as described earlier (18). Details of other methods used in this study, including RT-PCR (Table S3), Western blot, CysLT enzyme immunoassay, ABR analysis, immunohistochemistry, cytocochleogram, SEM, and RNA-seq profiling are described in *SI Materials and Methods*.

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1. Ohlemiller KK (2008) Recent findings and emerging questions in cochlear noise injury. *Hear Res* 245(1-2):5–17.
2. Weichbold V, Holzer A, Newesely G, Stephan K (2012) Results from high-frequency hearing screening in 14- to 15-year old adolescents and their relation to self-reported exposure to loud music. *Int J Audiol* 51(9):650–654.
3. Henderson E, Testa MA, Hartnick C (2011) Prevalence of noise-induced hearing-threshold shifts and hearing loss among US youths. *Pediatrics* 127(1):e39–e46.
4. Oishi N, Schacht J (2011) Emerging treatments for noise-induced hearing loss. *Expert Opin Emerg Drugs* 16(2):235–245.
5. Henderson D, Bielefeld EC, Harris KC, Hu BH (2006) The role of oxidative stress in noise-induced hearing loss. *Ear Hear* 27(1):1–19.
6. Tornabene SV, Sato K, Pham L, Billings P, Keithley EM (2006) Immune cell recruitment following acoustic trauma. *Hear Res* 222(1-2):115–124.
7. Sautter NB, Shick EH, Ransohoff RM, Charo IF, Hirose K (2006) CC chemokine receptor 2 is protective against noise-induced hair cell death: Studies in CX3CR1(+/GFP) mice. *J Assoc Res Otolaryngol* 7(4):361–372.
8. Ogawa Y, Calhoun WJ (2006) The role of leukotrienes in airway inflammation. *J Allergy Clin Immunol* 118(4):789–798, quiz 799–800.
9. Singh RK, Gupta S, Dastidar S, Ray A (2010) Cysteinyl leukotrienes and their receptors: Molecular and functional characteristics. *Pharmacology* 85(6):336–349.
10. Laidlaw TM, Boyce JA (2012) Cysteinyl leukotriene receptors, old and new; implications for asthma. *Clin Exp Allergy* 42(9):1313–1320.
11. Massoumi R, Sjölander A (2007) The role of leukotriene receptor signaling in inflammation and cancer. *ScientificWorldJournal* 7:1413–1421.
12. Le Prell CG, Yamashita D, Minami SB, Yamasoba T, Miller JM (2007) Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. *Hear Res* 226(1-2):22–43.
13. Yamashita D, Jiang HY, Schacht J, Miller JM (2004) Delayed production of free radicals following noise exposure. *Brain Res* 1019(1-2):201–209.
14. Shi X, Han W, Yamamoto H, Omelchenko I, Nuttall A (2007) Nitric oxide and mitochondrial status in noise-induced hearing loss. *Free Radic Res* 41(12):1313–1325.
15. Zhao R, et al. (2011) Pranlukast attenuates hydrogen peroxide-induced necrosis in endothelial cells by inhibiting oxygen reactive species-mediated collapse of mitochondrial membrane potential. *J Cardiovasc Pharmacol* 57(4):479–488.
16. Otunctemur A, et al. (2013) Protective effect of montelukast which is cysteinyl-leukotriene receptor antagonist on gentamicin-induced nephrotoxicity and oxidative damage in rat kidney. *Ren Fail* 35(3):403–410.
17. Becher UM, et al. (2011) Inhibition of leukotriene C4 action reduces oxidative stress and apoptosis in cardiomyocytes and impedes remodeling after myocardial injury. *J Mol Cell Cardiol* 50(3):570–577.
18. Park JS, et al. (2012) Pravastatin attenuates noise-induced cochlear injury in mice. *Neuroscience* 208:123–132.
19. Crow JP, Beckman JS (1995) Reactions between nitric oxide, superoxide, and peroxynitrite: Footprints of peroxynitrite in vivo. *Adv Pharmacol* 34:17–43.
20. Hu BH, et al. (2012) Metalloproteinases and their associated genes contribute to the functional integrity and noise-induced damage in the cochlear sensory epithelium. *J Neurosci* 32(43):14927–14941.
21. Kim EM, Hwang O (2011) Role of matrix metalloproteinase-3 in neurodegeneration. *J Neurochem* 116(1):22–32.
22. Michel O, Matthias R (1992) Effects of prostaglandin E2 on the fluctuating hearing loss in Ménière's disease. *Auris Nasus Larynx* 19(1):7–16.
23. Ogawa K, Takei S, Inoue Y, Kanzaki J (2002) Effect of prostaglandin E1 on idiopathic sudden sensorineural hearing loss: A double-blinded clinical study. *Otol Neurotol* 23(5):665–668.
24. Hori R, et al. (2009) Prostaglandin E receptor subtype EP4 agonist protects cochleae against noise-induced trauma. *Neuroscience* 160(4):813–819.
25. Al-Amran FG, Hadi NR, Hashim AM (2013) Cysteinyl leukotriene receptor antagonist montelukast ameliorates acute lung injury following haemorrhagic shock in rats. *Eur J Cardiothorac Surg* 43(2):421–427.
26. Daglar G, et al. (2009) Effect of montelukast and MK-886 on hepatic ischemia-reperfusion injury in rats. *J Surg Res* 153(1):31–38.
27. Erşahin M, et al. (2012) Montelukast inhibits caspase-3 activity and ameliorates oxidative damage in the spinal cord and urinary bladder of rats with spinal cord injury. *Prostaglandins Other Lipid Mediat* 99(3-4):131–139.
28. Fang SH, et al. (2009) Pranlukast attenuates ischemia-like injury in endothelial cells via inhibiting reactive oxygen species production and nuclear factor-kappaB activation. *J Cardiovasc Pharmacol* 53(1):77–85.
29. Hagar HH, Abd El Tawab R (2012) Cysteinyl leukotriene receptor antagonism alleviates renal injury induced by ischemia-reperfusion in rats. *J Surg Res* 178(1):e25–e34.
30. Bergeron MJ, Clémençon B, Hediger MA, Markovich D (2013) SLC13 family of Na^+-coupled di- and tri-carboxylate/sulfate transporters. *Mol Aspects Med* 34(2-3):299–312.
31. Ichimiya I, Yoshida K, Hirano T, Suzuki M, Mogi G (2000) Significance of spiral ligament fibrocytes with cochlear inflammation. *Int J Pediatr Otorhinolaryngol* 56(1):45–51.
32. Santi PA, Larson JT, Furcht LT, Economou TS (1989) Immunohistochemical localization of fibronectin in the chinchilla cochlea. *Hear Res* 39(1-2):91–101.
33. Satoh H, Kawasaki K, Kihara I, Nakano Y (1998) Importance of type IV collagen, laminin, and heparan sulfate proteoglycan in the regulation of labyrinthine fluid in the rat cochlear duct. *Eur Arch Otorhinolaryngol* 255(6):285–288.
34. Tsuprun V, Santi P (2001) Proteoglycan arrays in the cochlear basement membrane. *Hear Res* 157(1-2):65–76.
35. Nakagawa T (2011) Roles of prostaglandin E2 in the cochlea. *Hear Res* 276(1-2):27–33.
36. Nakashima T, et al. (2003) Disorders of cochlear blood flow. *Brain Res Brain Res Rev* 43(1):17–28.
37. Jung TT, et al. (1992) Effect of exogenous arachidonic acid metabolites applied on round window membrane on hearing and their levels in the perilymph. *Acta Otolaryngol Suppl* 493:171–176.
38. Tintinger GR, Feldman C, Theron AJ, Anderson R (2010) Montelukast: More than a cysteinyl leukotriene receptor antagonist? *ScientificWorldJournal* 10:2403–2413.
39. Mamedova L, et al. (2005) CysLT1 leukotriene receptor antagonists inhibit the effects of nucleotides acting at P2Y receptors. *Biochem Pharmacol* 71(1-2):115–125.
40. Lau WK, Chow AW, Au SC, Ko WH (2011) Differential inhibitory effects of CysLT(1) receptor antagonists on P2Y(6) receptor-mediated signaling and ion transport in human bronchial epithelia. *PLoS ONE* 6(7):e22363.
41. O'Keeffe MG, Thorne PR, Housley GD, Robson SC, Vlajkovic SM (2010) Distribution of NTPDase5 and NTPDase6 and the regulation of P2Y receptor signalling in the rat cochlea. *Purinergic Signal* 6(2):249–261.
42. Muñoz DJ, Kendrick IS, Rassam M, Thorne PR (2001) Vesicular storage of adenosine triphosphate in the guinea-pig cochlear lateral wall and concentrations of ATP in the endolymph during sound exposure and hypoxia. *Acta Otolaryngol* 121(1):10–15.
43. Piazza V, Ciubotaru CD, Gale JE, Mammano F (2007) Purinergic signalling and intercellular Ca2+ wave propagation in the organ of Corti. *Cell Calcium* 41(1):77–86.