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COCHLEAR PROTECTION FROM ACOUSTIC INJURY BY INHIBITORS OF p38 MITOGEN-ACTIVATED PROTEIN KINASE AND SEQUESTOSOME 1 STRESS PROTEIN

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Abstract—This study evaluated the protective role of p38 mitogen-activated protein kinase (p38 MAPK) inhibitors and sequestosome 1 (Sqstm1/A170/p62), a stress-induced signal modulator, in acoustic injury of the cochlea in mice. Two weeks after the exposure of mice to acoustic stress, threshold shifts of the auditory brainstem response (ABR) from the pre-exposure level and hair cell loss were evaluated. The activation of p38 MAPK was observed in cochlea by immunostaining 4 h after acoustic stress. To examine the role of p38 MAPK in tissue injury, its inhibitors were i.p. injected into male wild-type C57BL mice before the acoustic overexposure. The inhibitors SB202190 and SB203580 but not the inactive analogue SB202474 dose-dependently decreased the auditory threshold shift and outer hair cell loss induced by acoustic overexposure, suggesting the involvement of p38 MAPK in ototoxicity. We found that acoustic overexposure induced the up-regulation of *Sqstm1* mRNA expression in the cochlea of wild-type mice and that SQSTM1-deficient mice exhibited an enhanced ABR threshold shift and hair cell loss, suggesting a role of SQSTM1 in the protection of tissue from acoustic stress.

Key words: p38 MAPK, sequestosome 1, p62, A170, acoustic injury, cochlea.

Damage caused by acoustic overstimulation occurs primarily in the cochlea. Mechanosensory hair cells in the cochlea are susceptible to apoptotic death in response to exposure to ototoxic drugs and acoustic stress. The earliest signs of change induced by acoustic overexposure are often observed in outer hair cells (Boettcher et al., 1992; Davis et al., 1989; Hamernik et al., 1989). Hair cell loss is primarily observed in the first row of outer hair cells 2 weeks after acoustic overexposure (Yamasoba et al., 1998), although the precise mechanism of hair cell death is unknown.

Mitogen-activated protein kinase (MAPK) is an important intracellular signal transduction system involved in a series of physiological processes such as cell growth, differentiation, and apoptotic cell death. The c-Jun NH2-terminal kinase (JNK) and p38 MAPK pathways are strongly stimulated by inflammatory cytokines and environmental stress including oxidative stress, osmotic stimuli, and heat shock (Juntilla et al., 2008; Matsuzawa and Ichijo, 2008). It has been reported that MAPK modulates apoptosis, development, inflammation, and transformation, responding to various types of stress (Tibbles and Woodgett, 1999; Borsello et al., 2003). Recent studies suggested that MAPK is involved in the aminoglycoside-induced apoptosis of cochlear outer hair cells. The administration of aminoglycoside generates reactive oxygen species, which in turn activates MAPK. Wei et al. (2005) reported that p38 MAPK was activated after the administration of gentamicin, and that minocyclin reduced the cell death of outer hair cells through inhibiting the p38 MAPK pathway. Based on the previous findings, it is possible that acoustic overexposure activates p38 MAPK to aggravate acoustic injury.

The cytoplasmic signal adaptor protein designated sequestosome 1 (SQSTM1/ A170/p62/ZIP) plays a role in modulating signal transduction via membrane receptors (Moscat et al., 2007). It interacts with different protein kinases, such as tyrosine kinase $p56^{lck}$ (Joung et al., 1996) and PKCζ (Puls et al., 1997), and affects the activities of ERK (Rodriguez et al., 2006) and p38 MAPK (Sudo et al., 2000; Kawai et al., 2008; Sugimoto et al., 2009). We recently showed that SQSTM1-deficient vascular smooth muscle cells caused enhanced p38 MAPK activation upon serum stimulation *in vitro* (Sugimoto et al., 2009), suggesting that SQSTM1 suppresses the activation of p38 MAPK. We previously reported that SQSTM1/A170 is an oxidative stress- or electrophile-inducible protein (Ishii et al., 1996), regulated by the redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2) (Ishii et al., 2000). Nrf2 activates the ARE/EpRE (antioxidant/electrophile responsive element)-mediated expression of phase II detoxifying and antioxidant genes (Ishii et al., 2000).

In this study, we demonstrated that acoustic overexposure activated p38 MAPK in the cochlea, and its inhibitors suppressed tissue injury caused by the stress. Furthermore, we showed for the first time that *Sqstm1* is up-regulated by acoustic stress and that SQSTM1 deficiency aggravated injury to the cochlea.

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Abbreviations: ABR, auditory brainstem response; JNK, c-Jun NH2-terminal kinase; Nrf2, NF-E2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase.

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EXPERIMENTAL PROCEDURES

Animals

C57BL/6J mice at 2 months of age were used in this study. Male Sqstm1 knockout mice constructed by employing gene-targeting procedures (Komatsu et al., 2007) with a C57BL/6J background were used in this study. The animals were maintained under a normal day/night cycle and had free access to food and water. The care and use of animals were approved by the Animal Research Committee and Safety Committee for Gene Recombination Research of the University of Tsukuba.

Measurement of auditory brainstem response (ABR)

Mice were anesthetized with the i.p. injection of 50 mg/kg pentobarbital sodium (Dainihon Pharmaceuticals Inc., Osaka, Japan). Positive, negative, and ground electrodes were inserted s.c. at the vertex, mastoid, and back, respectively (Tabuchi et al., 2005; Murashita et al., 2006). ABR responses were evoked by tone bursts of pure tones (rise and fall times, 1 ms; duration, 10 ms). Evoked responses were filtered with a band pass of 200 to 3000 Hz and averaged with 1000 sweeps using a signal processor (Synax, 2100, NEC, Tokyo, Japan). The sound intensity was varied in 5-dB steps. The hearing threshold was defined as the lowest intensity yielding the consistent appearance of at least one ABR peak. ABR threshold shifts were examined before, 1, and 2 weeks after acoustic exposure.

Acoustic overexposure

The mice were subjected to a 4-kHz pure tone of 128 dB SPL for 4 h through an open field system inside a sound-exposure chamber (Type 4212, Brüel and Kjaer, Copenhagen, Denmark). The sound intensity was measured at multiple locations within the sound chamber using an SPL meter (NA-60, RION, Tokyo, Japan) to ensure uniformity of the exposure stimuli, and the sound intensity of the exposure stimuli was confirmed to vary by a maximum of 1 dB (Tabuchi et al., 2005; Uemaetomari et al., 2005).

Evaluation of outer hair cell loss

The mice were sacrificed under deep anesthesia 2 weeks after acoustic overexposure. Cardiac perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Cochleae were then removed and immersed in the same fixative for 8 h. After fixation, whole mounts of the organ of Corti were prepared as surface preparations, and the nuclei of hair cells were stained with Propidium Iodide (PI, Molecular Probes Inc., OR, USA) (Uemaetomari et al., 2005; Murashita et al., 2006).

All specimens were thoroughly inspected under a laser confocal microscope from the apex to base (TCS 4D, Leica Microsystems, Wetzlar, Germany). The pattern of hair cell loss in the present model of acoustic injury was described in detail in our previous report (Uemaetomari et al., 2005). Namely, the loss of outer hair cells was observed maximally at the 3.96 mm region from the apex, although the hair cell loss spread from 50% to 80% region. In this study, we compared hair cell loss and activation of p38 MAPK among the groups at the 3.96 mm region.

Detection of p38 MAPK activation after acoustic overexposure

The mice were sacrificed under deep anesthesia immediately after the termination of 4 h acoustic overexposure. After cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, cochleae were removed and prepared for surface preparations. The specimens were treated with 1.2% hydrogen peroxidase for 20 min. After blocking non-specific staining with 5% normal serum, the specimens were stained with an antibody for activated p38 MAPK (Molecular Probes Inc., OR, USA) following the manufacture's protocol. After the specimens were rinsed with 0.1 M PBS three times, a fluorescein isothiocyanate (FITC)-conjugated second antibody was added. In addition, phalloidin was stained with a conjugated Alexa Fluro probe (1:100, Molecular probes Inc., Carlsbad, CA, USA) at room temperature for 1 h. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and cuticular plates of hair cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Animals were sacrificed before and just after acoustic overexposure. Cochleae were dissected under a microscope, and the organ of Corti was removed for the RT-PCR study. Total RNAs were isolated using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan) and purified with a High Pure RNA Tissue Kit (Roche Diagnostics, Indianapolis, IN, USA). The cDNAs were synthesized using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). Real-time RT-PCR was carried out with SYBR Premix ExTaq II (TaKaRa, Tokyo, Japan) using the ABI-PRISM 7000 Sequence Detector System (Applied Biosystems, Carlsbad, CA, USA). The PCR thermocycling parameters were 95 °C for 10 s, and 40 cycles of 95 °C for 5 s, and 60 °C for 31 s. The primers were as follows: Sqstm1, forward: 5′-CCTCAGCCCTCTAGGCATTG-3′, reverse: 5′-CTGTGCCTGTGCTGGAACTTT-3′, Nqo1, forward: 5′-GGGTCGTCTTGGCAACCA-3′, reverse: 5′-CAGATGTTGAGGGAGGATCGTAA-3′, cyclophilin, forward: 5′-TGGAGAGCACCAAGACAGACA-3′, reverse: 5′-TGCCGGAGTCGACAATGAT-3′. Each sample was assayed in triplicate and the expression levels were normalized to that of cyclophilin.

Drug administration

SB202190 and SB203580 (selective inhibitors of p38 MAPK), and SB202474 (an inactive analogue of these inhibitors) were used in this study. These agents were dissolved in 0.5 ml/body of 10% dimethyl-sulfoxide (DMSO). All drugs were administered s.c. in the back of the animals immediately before the onset of acoustic overexposure. Control mice received 10% dimethyl-sulfoxide following the same schedule.

Experimental design

For ABR testing and evaluating outer hair cell loss after acoustic overexposure, the animals were assigned to the following six groups and subjected to acoustic overexposure:

(1) SB202190 group (n=20): mice in this group were treated with 0.1 (n=5), 0.3 (n=5), 1 (n=5), or 3 (n=5) mg/kg SB202190.
(2) SB203580 group (n=20): mice in this group were treated with 0.1 (n=5), 0.3 (n=5), 1 (n=5), or 3 (n=5) mg/kg SB203580.
(3) SB202474 (n=5): mice in this group were treated with 3 mg/kg SB202474.
(4) DMSO-administered control group (n=5): mice in this group were treated only with 10% dimethyl-sulfoxide. This group was set up as the control for groups (1), (2), and (3).
(5) Sqstm1 knockout group (n=6): the Sqstm1 knockout mice were subjected to acoustic overexposure.
(6) Nontreated control group (n=6): these animals were the control for the Sqstm1 knockout group, and were subjected to acoustic overexposure without any medication.

In addition to the above-mentioned 62 animals, the activation of p38 MAPK was immunohistochemically examined using 10 animals. In addition, the RT-PCR study was conducted using nine animals.

Data analysis

All data are presented as means±SEM. ABR threshold shifts and missing hair cell rates were evaluated using two-way factorial ANOVA. A *P*-value of less than 0.05 was considered significant.

RESULTS

Acoustic overexposure activated p38 MAPK

Cochleae were stained with antibody reactive with phosphorylated/activated p38 MAPK to examine whether or not acoustic overexposure activates p38 MAPK. p38 MAPK was not activated in the control cochleae, which were not subjected to acoustic overexposure (Fig. 1A, C, E). However, cells stained for activated p38 MAPK significantly increased at the 3.96 mm region from the apex in animals sacrificed at the termination of the 4-h acoustic overexposure (Fig. 1B, D, F, G, two-way factorial ANOVA: $P<0.01$).

p38 MAPK inhibitors ameliorated the ABR threshold shift and hair cell loss induced by acoustic overexposure

The mice were subjected to acoustic overexposure of 128 dB SPL for 4 h. Fig. 2 shows the effects of the p38 MAPK inhibitors SB202190 and SB203580 on the ABR threshold shifts 2 weeks after acoustic overexposure. Administration of 1 and 3 mg/kg SB202190 significantly reduced the ABR threshold shift compared with the control animals both 1 and 2 weeks after acoustic overexposure (two-way factorial ANOVA and Scheffe's test: $P<0.01$), although 0.1 and 0.3 mg/kg SB202190 failed to alleviate acoustic injury (Scheffe's test: $P>0.05$). Similarly, SB203580 also ameliorated the ABR threshold shift at 1 and 3 mg/kg both 1 and 2 weeks after acoustic overexposure (two-way facto-

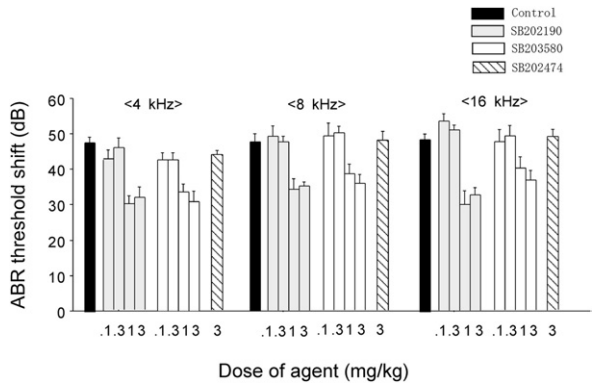

Fig. 2. The ABR threshold shift at 2 weeks after acoustic overexposure. p38 MAPK inhibitors SB202190 and SB203580 decreased the ABR threshold shift at doses of 1 and 3 mg/kg. However, an inactive analogue of the p38 MAPK inhibitors (SB202474) did not alleviate the ABR threshold shift.

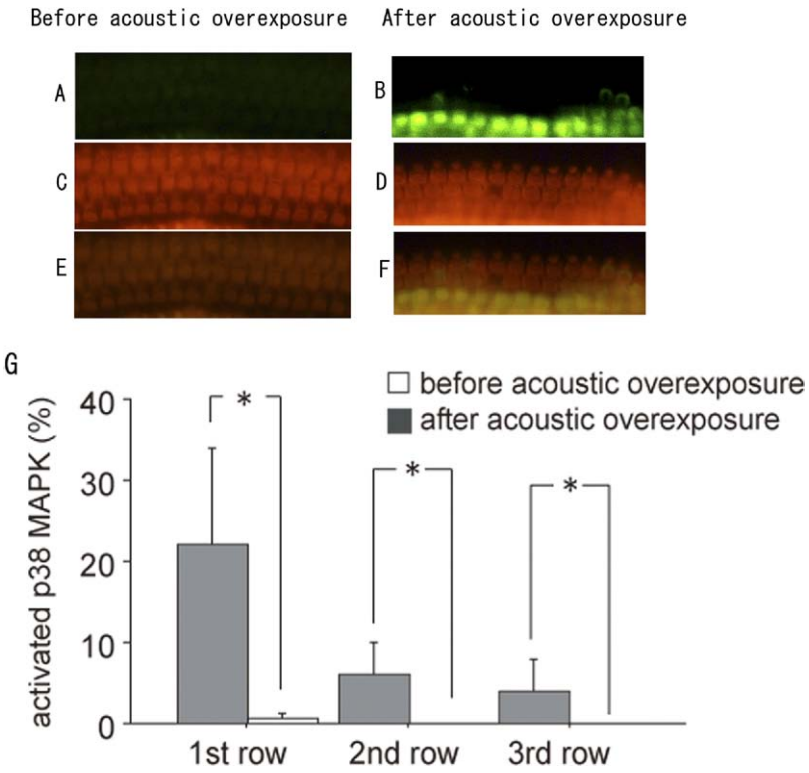

Fig. 1. (A–D) Representative fluorescence micrographs of p38 MAPK activation (A and B: FITC, green) and outer hair cells (C and D: Alexa Fluro probe, red) at the 3.96 mm region from the apex (E and F: composite micrographs of FITC and Alexa Fluro staining). p38 MAPK was not activated in hair cells before acoustic overexposure (A and E). Acoustic overexposure for 4 h activated p38 MAPK in the cochlea (B and F). (G) Quantitative analysis of p38 MAPK activation at the 3.96 mm region from the apex (%). Outer hair cells showing p38 MAPK activation significantly increased after acoustic overexposure.

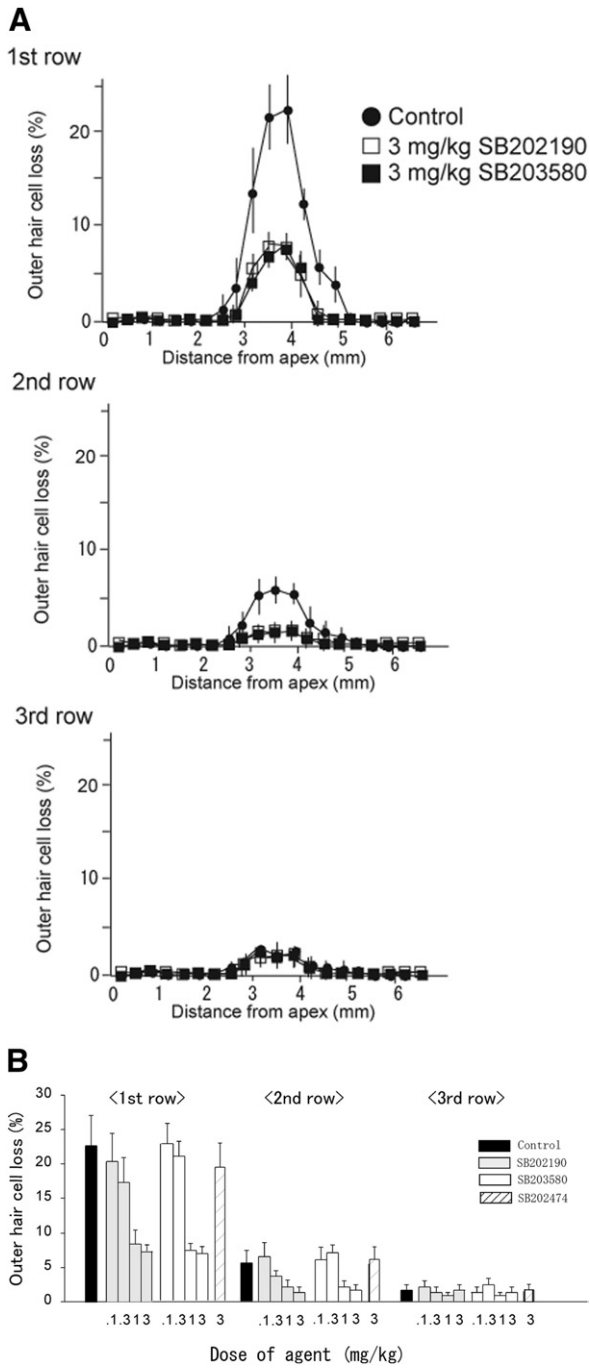

Fig. 3. (a) cytocochleogramas 2 weeks after acoustic overexposure. Hair cell loss was observed at the 2.97 to 4.95 mm region from the apex. Hair cell loss was reduced by administration of 3 mg/kg SB202190 or 3 mg/kg SB203580. Outer hair cell loss (%) 2 weeks after acoustic overexposure. (b) Outer hair cell loss induced by acoustic overexposure was evident in the first row of the outer hair cells. p38 MAPK inhibitors (SB202190 and SB203580) decreased outer hair cell loss at doses of 1 and 3 mg/kg SB202474 did not reduce outer hair cell loss.

rial ANOVA: $P<0.01$, Scheffe's test: $P<0.01$ at 1 and 3 mg/kg and $P>0.05$ at 0.1 and 0.3 mg/kg). In contrast to the protective effects observed in the animals treated with SB202190 or SB203580, 3 mg/kg SB202474, an inactive analogue, did not exhibit any protective effect (two-way factorial ANOVA).

Hair cell loss induced by acoustic overexposure was examined 2 weeks after the exposure. The cytocochleograms of hair cell loss are shown in Fig. 3a. Three mg/kg SB202190 and 3 mg/kg SB203580 reduced hair cell loss as compared with the control group. Fig. 3b shows hair cell loss in the 3.96 mm region from the apex, where the maximum hair cell loss was observed. One and 3 mg/kg SB202190 significantly reduced outer hair cell loss compared with the control group (two-way factorial ANOVA and Scheffe's test: $P<0.01$). However, 0.1 and 0.3 mg/kg SB202190 did not exhibit any protective effect (Scheffe's test: $P>0.05$). In agreement with ABR testing, SB203580 reduced hair cell loss at 1 and 3 mg/kg (two-way factorial ANOVA and Scheffe's test: $P<0.01$ at 1 and 3 mg/kg and $P>0.05$ at 0.1 and 0.3 mg/kg). SB202474 did not exhibit any protective effect.

Acoustic overexposure increased expression of Sqstm1 mRNA

The relative mRNA expression of *Sqstm1* and *Nqo1* to *cyclophilin* was examined in the wild-type mice without treatment ($n=5$) and after ($n=4$) 4-h acoustic overexposure. As shown in Fig. 4, the expression level of *Nqo1* mRNA did not change on overexposure. However, mRNA expression of *Sqstm1* was significantly elevated after compared with that before acoustic overexposure (one-way ANOVA: $P<0.05$ for Sqstm1 expression).

Deficiency of Sqstm1 made the cochlea vulnerable to acoustic injury

Knockout of *Sqstm1* gene itself did not affect the ABR threshold (Fig. 5A, two-way factorial ANOVA). Namely, *Sqstm1* deletion did not affect the hearing level. Fig. 5B shows the ABR threshold shift 2 weeks after acoustic overexposure in the *Sqstm1* knockout mice. The ABR threshold shift significantly increased in the *Sqstm1* knockout mice compared with the nontreated control group (two-way factorial ANOVA: $P<0.01$). As shown in Fig. 6a, b, the disruption of *Sqstm1* increased outer hair cell loss at the

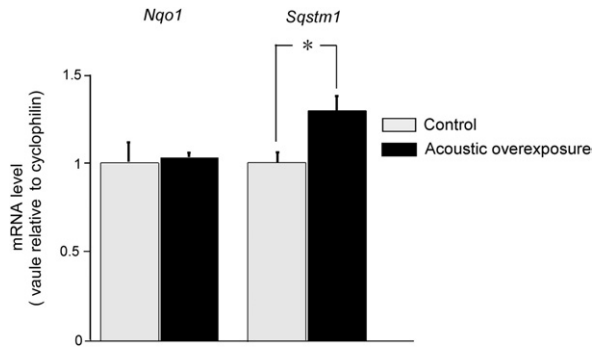

Fig. 4. mRNA levels of Sqstm1 and NQO1 relative to cyclophilin. The animals were subjected to acoustic overexposure, and mRNA expression was examined. The relative level of Sqstm1 mRNA was elevated after acoustic overexposure.

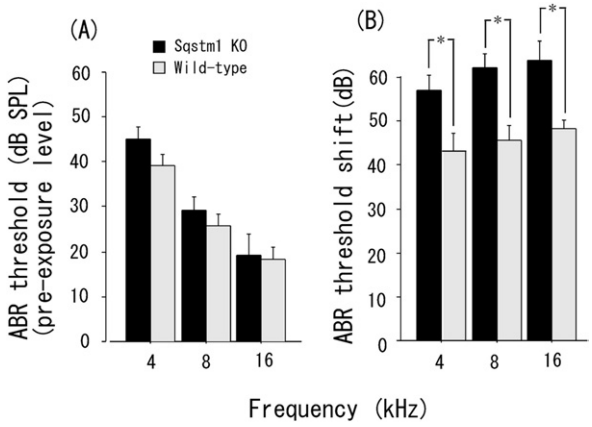

Fig. 5. (A) Knockout of Sqstm1 did not affect the ABR threshold at 2 months of age. (B) The ABR threshold shift induced by acoustic overexposure increased in the *Sqstm1* knockout as compared with the wild-type mice (two-way factorial ANOVA: * $P<0.01$).

3.96 mm region from the apex 2 weeks after acoustic overexposure (two-way factorial ANOVA: $P<0.01$).

DISCUSSION

In this study, we have shown for the first time the involvement of p38 MAPK in cochlear injury *in vivo* after acoustic overexposure. This is based on the fact that p38 MAPK is activated by acoustic overexposure in hair cells (Fig. 1) and that administration of p38 MAPK inhibitors into mice specifically suppress the toxicity (Figs. 2 and 3). MAPKs play a critical role in cell signaling and gene expression. p38 MAPK is activated by phosphorylation (by upstream kinase MKK3/MMK6) and transduce a broad range of extracellular stimuli into diverse intracellular responses such as apoptosis. p38 MAPK phosphorylation/activation in hair cells was also reported in gentamicin ototoxicity by Wei et al. (2005). They used cultured cochlear explants from rats and suggested that minocycline blocks gentamicin-induced hair cell loss by inhibition of p38 activation (Wei et al., 2005).

In this study, we also demonstrated a protective role of the stress-induced protein SQSTM1 in acoustic injury of the cochlea. SQSTM1-deficient mouse exhibited an enhanced ABR threshold shift and hair cell loss 2 weeks after acoustic overexposure. We observed the up-regulation of *Sqstm1* gene expression in wild-type mice after acoustic stress (Fig. 4), although we could not detect an increase in SQSTM1 protein levels due to limited proteins from the cochlea. Even though the transcription factor Nrf2 enhances *Sqstm1* gene expression in the presence of various stresses (Ishii et al., 2000), Nrf2 may not be directly involved in *Sqstm1* expression after acoustic overexposure, since another Nrf2 target gene, *Nqo1*, was not up-regulated (Fig. 4). Our results indicate that SQSTM1 could protect hair cells from acoustic stress. It is highly speculative at this moment, but we suggest a possible idea that SQSTM1 suppresses the activation of p38 MAPK induced by the stress, since we observed that presence of SQSTM1 suppressed activation of p38 MAPK in vascular

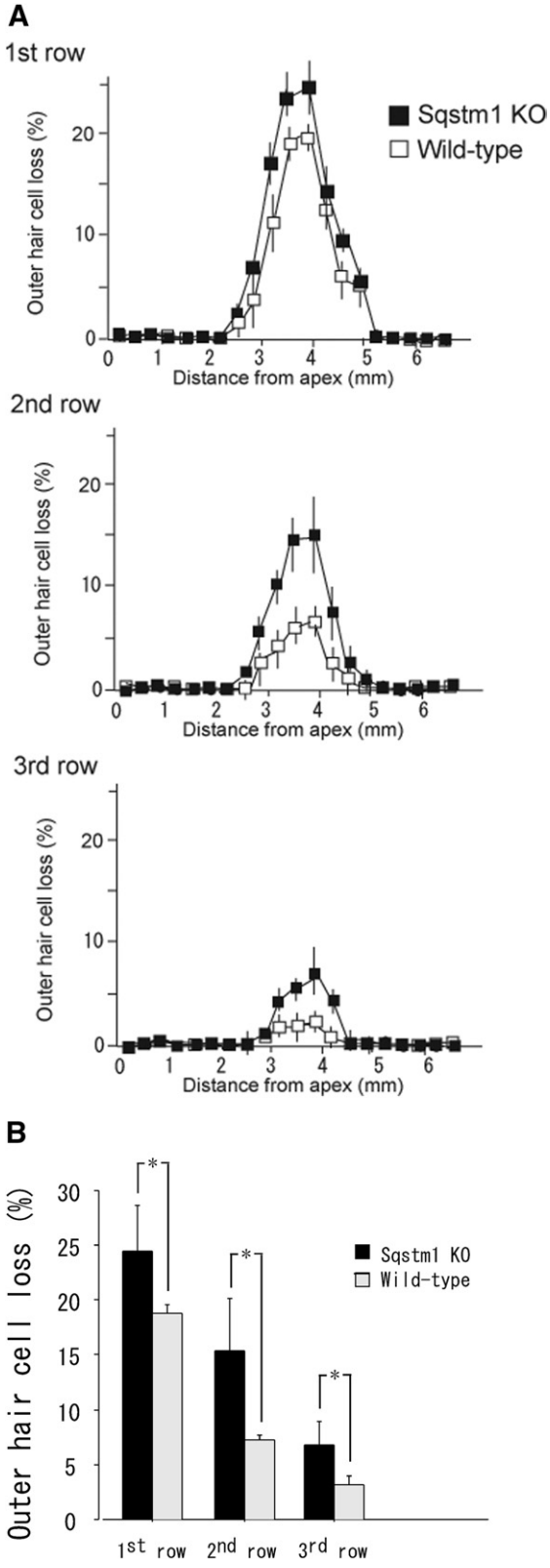

Fig. 6. (a) Cytocochleogram of missing outer hair cells in the Sqstm1 knockout and wild-type mice. (b) Knockout of *Sqstm1* increased outer hair cell loss at the 3.96 mm region from the apex (two-way factorial ANOVA: * $P<0.01$).

smooth muscle cells upon stimulation with fetal bovine serum (Sugimoto et al., 2009). We are further characterizing the precise role and activation mechanism of SQSTM1 in the protection of hair cells from acoustic injury.

The ABR threshold shift in this study showed a flat pattern of the frequency-threshold shift curve. Maximal hearing loss is reported to typically occur in the frequency 1/2 octave above the frequency of acoustic overexposure (half-octave shift theory). We used a 4 kHz pure tone of 128 dB SPL to induce acoustic injury, and this intense sound induced hair cell loss in a wide area of the cochlea, as shown in the cytocochleograms. Hair damage might have occurred in a wider cochlear area than the area of hair cell loss. The flat pattern of threshold shifts was observed probably because the intensity of acoustic overexposure is high in this study. Furthermore, Canlon (1988) showed that as intensities to induce acoustic injury increased, the maximum threshold shift was observed in higher frequencies than speculated by the half-octave shift theory.

CONCLUSION

Our study indicates the role of p38 MAPK activation in hair cell injury caused by acoustic overexposure. We show for the first time the protective role of the stress protein SQSTM1 in ototoxicity. Further precise studies on these two key factors will elucidate the generation mechanism(s) of acoustic injury to the cochlea, and will help establish a clinical approach to protect mechanosensory hair cells from stress.

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