The Non-steroidal Anti-inflammatory Drugs Protect Mouse Cochlea against Acoustic Injury

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Acoustic injury is a common cause of hearing loss for people in industrial societies. Cyclooxygenase (COX) and lipoxygenase (LOX) are two important enzymes involved in arachidonic acid metabolism. Two COX isozymes are characterized, COX-1 and COX-2, that differ in terms of regulatory mechanisms of expression. Although COX-1, COX-2, and LOX are expressed in cochlea, their roles played in cochlear acoustic injury have not fully been evaluated. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit either COX or LOX, or both. This study evaluated the effects of NSAIDs on the functional recovery of the cochlea after acoustic injury. Mice were exposed to a 4-kHz pure tone of 128 dB SPL (sound pressure level) for 4 hours and received one of the following drugs for two weeks after acoustic overexposure: indomethacin (COX-1 inhibitor), meloxicam, SC58125, and CAY10404 (COX-2 inhibitors), and nordihydroguaiaretic acid (LOX inhibitor). The hearing ability was evaluated using an auditory brainstem response (ABR) before and after overexposure. The ABR threshold shifts, defined as subtraction between ABR thresholds before and after overexposure, were compared among the control and the medication groups at one and two weeks after acoustic overexposure. Treatment of mice with either indomethacin or nordihydroguaiaretic acid decreased the ABR threshold shifts after overexposure, indicating that COX-1 and LOX inhibitors exhibited protective effects against acoustic injury. In contrast, COX-2 inhibitors, meloxicam, SC58125, and CAY10404, showed no noticeable effects on the ABR threshold shifts. These findings suggest that COX-1 and LOX are involved in the pathogenesis of acoustic injury in cochlea. ——— cyclooxygenase; lipoxygenase; non-steroidal anti-inflammatory drugs; cochlea; acoustic injury.

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Exposure to high levels of sound pressure causes hearing loss by damaging the cochlea, particularly inducing loss of sensory hair cells (reviewed by Tabuchi et al., 2007). Acoustic injury is one of the main sources of hearing loss for people in industrial societies. In addition, the

noisy sounds of everyday life make acoustic injury a predicament that may affect all members of society.

Prostaglandins (PGs) and leukotrienes (LTs) are generated through the arachidonic cascade. In the first step of the arachidonic cascade, arachi-

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donic acid (AA) is generated from membrane phospholipid precursors by phospholipase A₂. The next stage of this cascade has two main pathways: the cyclooxygenase (COX) pathway that produces PGs and thromboxanes (TXs), and the lipoxygenase (LOX) pathway that produces LTs.

COX is currently categorized into two isozymes, COX-1 and COX-2 (Gierse et al. 1996; Gilroy et al. 1998; Vane et al. 1998; Reddy et al. 1999). COX-1 is constitutively expressed in most tissues of mammals, whereas COX-2 is not detected in normal tissues with the exception of the kidney and central nervous system (Lopez-Garcia and Laird 1998), and is induced in response to various stimuli, such as cytokines, bacterial lipopolysaccharide and growth factors. (Barnett et al. 1994; Blanco et al. 1999)

Non-steroidal anti-inflammatory drugs (NSAIDs) block the COX and/or LOX pathways as competitive inhibitors. COX inhibitors and LOX inhibitors inhibit the production of PGs and LTs, respectively. PGs and/or LTs are known to be inflammatory and pyretogenic substances, and thus some of NSAIDs are clinically used as analgesics and antipyretics. Although NSAIDs are widely used clinically, the effects of NSAIDs on acoustic injury of the cochlea have attracted only little attention. To the best of our knowledge, only the effects of diclofenac (Lamm and Arnold 1998) and salicylate (Kopke et al. 2000) on acoustic injury have been reported.

In this study, we examined the effects of NSAIDs on acoustic injury of the cochlea. We used selective inhibitors of COX-1, COX-2 and LOX. Indomethacin is a COX-1 preferential inhibitor (Barnett et al. 1994; Blanco et al. 1999). Meloxicam (Pairet et al. 1998; Blanco et al. 1999), SC58125 (Anderson et al. 1996) and CAY10404 (Katada et al. 2005) are defined as COX-2 inhibitors. Nordihydroguaiaretic acid (NDGA) was reported to be a selective LOX inhibitor (Mayer et al. 1986). The purpose of the present study was therefore to examine the effects of these NSAIDs on acoustic injury of the mouse cochlea and to clarify differences in contribution to the protective effect.

MATERIALS AND METHODS

Animals

Sixty ddY mice (8 weeks of age) were used in this study. The ddY mouse is one of the inbred strain mice that was established at the National Institute of Health of Japan. This strain is easy to maintain because of good fertility and growth. Therefore, it is frequently used as a subject for pharmacological and toxicological experiment in Japan. The animals were kept on a normal day/night cycle with free access to food. The care and use of the animals were approved by the Animal Experiment Committee of the University of Tsukuba.

Auditory brainstem response (ABR) testing

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Positive, negative and ground electrodes were inserted subcutaneously at the vertex, mastoid and back, respectively. Pure tone bursts (rise and fall times, 1 ms; duration, 10 ms) were used to evoke ABR. Evoked responses were amplified, filtered with a band pass of 200 to 3,000 Hz and averaged with 1,000 sweeps using a signal processor (Synax 1200, NEC, Tokyo, Japan). Sound intensity 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 from pre-exposure levels were examined one and two weeks after acoustic overexposure.

Acoustic overexposure

The mice were subjected to a 4 kHz pure tone of 128 dB SPL for 4 hours through an open field system inside a sound-exposure chamber (Type 4212, Brüel & Kjaer, Copenhagen, Denmark). Sound intensity was measured at multiple locations within the sound chamber using an SPL meter (NA-60, RION, Tokyo, Japan) to ensure the uniformity of exposure stimuli, and the sound intensity of exposure stimuli was confirmed to vary by a maximum of 1 dB (Uemaetomari et al. 2005; Murashita et al. 2006).

Histological examination

The mice were sacrificed under deep anesthesia two 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 hours. After fixation, whole mounts of the organ of Corti were dissected as surface preparations, and the nuclei of hair cells were

stained with propidium iodide (PI, Molecular Probes Inc., OR).

All specimens were thoroughly inspected under a laser confocal microscope (TCS 4D, Leica Microsystems, Wetzlar, Germany). The number of missing hair cells (absence of PI staining) was determined from the apex to the base in 0.33 mm segments and plotted as cytochromeographs.

Drug administration

Indomethacin, SC58125, CAY10404 and NDGA (Cayman chemical, Ann Arbor, MI) were dissolved in peanut oil (5 ml/kg), and meloxicam (BIOMOL, Plymouth Meeting, PA) in physiological saline solution (PSS, 5 ml/kg). All drugs were administered subcutaneously in the back of the animals. Drug administration started immediately after acoustic overexposure and was then administered twice a day during the two-week experimental period. Control mice received 5 ml/kg of PSS or peanut oil at the same time and schedule after acoustic overexposure. The concentration of each drug was decided based on the minimum concentration at which the drug inhibits PGE2 activity or pain. (Nakayama et al. 1998; Pairet et al. 1998; Katada et al. 2005)

Experimental design

Mice were randomly assigned to one of the following 7 treatment groups:

- (1) indomethacin group ($n = 10$): Mice in this group were treated with 5 mg/kg ($n = 5$) or 1 mg/kg ($n = 5$) indomethacin.
- (2) meloxicam group ($n = 10$): Mice in this group were treated with 50 mg/kg ($n = 5$) or 10 mg/kg ($n = 5$) meloxicam.
- (3) SC58125 group ($n = 5$): Mice in this group were treated with 10 mg/kg SC58125.
- (4) CAY10404 group ($n = 15$): Mice in this group were treated with 30 mg/kg ($n = 5$), 10 mg/kg ($n = 5$) or 3 mg/kg ($n = 5$) CAY10404.
- (5) NDGA group ($n = 10$): Mice in this group were treated with 100 mg/kg ($n = 5$) or 10 mg/kg ($n = 5$) NDGA.
- (6) PSS-treated control group ($n = 5$): Mice in this group were treated with 5 ml/kg PSS.
- (7) peanut oil-treated control group ($n = 5$): Mice in this group were treated with 5 ml/kg peanut oil.

Data analysis

All values are presented as mean $\pm$ standard deviation (s.d.) ABR threshold shifts. Percent of missing hair cells were evaluated using two-way factorial ANOVA. Scheffe test and Fisher's PLSD test were used as a post hoc test. A p value of less than 0.05 was considered significant.

RESULTS

ABR threshold shift after acoustic overexposure

The effect of indomethacin, a COX-1 inhibitor, on ABR threshold shifts one and two weeks after acoustic overexposure is shown in Fig. 1A-B. Indomethacin (5 mg/kg) significantly reduced the ABR threshold shift as compared with peanut oil-given control animals both one and two weeks after acoustic overexposure ($p < 0.01$), although indomethacin at a lower concentration (1 mg/kg) failed to alleviate acoustic injury at one or two weeks after acoustic overexposure ($p > 0.05$ at one and two weeks). As shown in Fig. 1C-D, NDGA (100 mg/kg), a LOX selective inhibitor, significantly decreased the ABR threshold shift both one and two weeks after acoustic overexposure (two-way factorial ANOVA: $p < 0.01$, Scheffe test: $p < 0.01$ in the 100 mg/kg group and $p > 0.05$ in the 10 mg/kg group).

The effects of meloxicam, SC58125 and CAY10404, COX-2 inhibitors, on the ABR threshold shifts induced by acoustic overexposure are shown in Fig. 2A-B, respectively. Meloxicam (50 mg/kg) slightly reduced the ABR threshold shift one week after acoustic overexposure (Fig. 2A: $p = 0.04$); however, there was no statistically significant difference in the ABR threshold shift between the meloxicam groups (10 or 50 mg/kg) and the peanut oil-given control group at two weeks after acoustic overexposure (Fig. 2B). As shown in Fig. 2C-F, SC58125 and CAY10404, which are more selective COX-2 inhibitors than meloxicam, did not have any effect on the ABR threshold shift at one or two weeks after acoustic overexposure.

Histological change by medication

Our previous reports clarified that acoustic

Fig. 1. The effects of COX-1 and LOX inhibitors on ABR threshold shifts.

ABR threshold shifts in the indomethacin group (A and B) and NDGA group (C and D) one week (A and C) and two weeks (B and D) after acoustic overexposure. The COX-1 and LOX inhibitors decreased ABR threshold shifts. Data was analyzed using a two-way factorial ANOVA, Scheffe test, $**p < 0.01$.

Fig. 2. The effects of COX-2 inhibitors on ABR threshold shifts.

ABR threshold shifts in the meloxicam group (A and B), SC58125 group (C and D), and CAY10404 group (E and F) one week (A, C, and E) and two weeks (B, D, and F) after acoustic overexposure. The COX-2 inhibitors did not decrease ABR threshold shifts. Data was analyzed using a two-way factorial ANOVA, Scheffe test, $*p < 0.05$.

Fig. 3. Profile of hair cell loss after acoustic overexposure.

Propidium iodide staining of the nuclei of hair cells (A) Representative micrograph of organ of Corti in 66% region from the apex. Note that acoustic overexposure induced hair cell loss as indicated by arrowheads (absence of staining of propidium iodide). Graphs showing hair cell loss in a 66% region from the apex by medication, (B) inner hair cells, (C) first row of outer hair cells, (D) second row of outer hair cells, and (E) third row of outer hair cells. Analysis of data was performed using a two-way factorial ANOVA, Fisher's PLSD, * $p < 0.05$. Scale bar: 0.1 mm.

overexposure of 128 dB SPL for 4 hours induced hearing loss and hair cell loss (Uemaetomari et al. 2005; Murashita et al. 2006). The maximum hair cell loss was observed in a 66% region from apex of the cochlea in this model (Fig. 3A: Uemaetomari et al. 2005; Murashita et al. 2006). Fig. 3 shows the percentages of hair cell loss in this region (B-E). Indomethacin (5 mg/kg) and NDGA (100 mg/kg) significantly reduced the hair

cell loss compared with the control group ($p < 0.05$), indicating that COX-1 and LOX inhibitors have the protective effect against acoustic injury.

DISCUSSION

To our knowledge, diclofenac (Lamm and Arnold 1998) and salicylate (Kopke et al. 2000) are the only two NSAIDs reported to protect the cochlea against acoustic injury. Although they are

COX-1 inhibitors, it has not been reported whether other NSAIDs, COX-2 and LOX inhibitors, also protect against acoustic injury. As NSAIDs are capable of blocking the COX and LOX pathways, the protective mechanism of the cochlea against acoustic injury might differ among the various categories of NSAIDs. We, therefore, tested several kinds of NSAIDs in acoustically injured mice and compared their protective effect. The present study demonstrated that indomethacin and NDGA also have protective effects on acoustic injury of the cochlea. Considering the selectivity of the drugs used in the present study, it is highly likely that COX-1 and LOX are involved in acoustic injury of the cochlea.

Although little is known about the mechanism of cochlea protection by NSAIDs, it is possible that NSAIDs have anti-inflammatory and anti-oxidant actions. As regards the anti-inflammatory action of NSAIDs, TXs and LTs are generated via COX and LOX pathways, respectively. Over-production of TXs and LTs is reported to be toxic to the cochlea (Jung et al. 1992; Umemura 1993); and it is possible that NSAIDs protect the cochlea against acoustic injury by inhibiting over-production of these metabolites. Regarding the anti-oxidant action, accumulated evidence suggests that reactive oxygen species (ROS) are produced by acoustic overexposure and that ROS play key roles in acoustic injury (Yamane et al. 1995; Yamasoba et al. 1998; Ohlemiller et al. 1999). Although the mechanisms generating ROS in the cochlea after acoustic overexposure have not been clarified. It has been suggested that xanthine oxidase (Seidman et al. 1993), mitochondria (Chance et al. 1979) and arachidonic cascade (Rieger et al. 2002) are generally associated with the production of ROS. AA is oxidized by COX to form PGG₂, which is subsequently transformed by hydroperoxidase to form PGH₂. As ROS are produced during the conversion of PGG₂ to PGH₂ (Yu and Wells 1995), ROS production is inhibited by COX inhibitors. Similarly, LOX oxidizes AA to form hydroperoxy-eicosatetraenoic acid (HPETE). Subsequently, HPETE is converted to hydroxy-eicosatetraenoic acid (HETE) by hydroperoxidase, and again ROS are produced

during the conversion of HPETE to HETE (Yu and Wells 1995). COX and LOX inhibitors thus block ROS production and these actions might account for the protective mechanism of COX and LOX inhibitors.

CONCLUSION

The present study demonstrates the protective effects of indomethacin and NDGA against acoustic injury, suggesting that COX-1 and LOX play key roles in this type of injury. Given that NSAIDs are widely used in humans, these anti-inflammatory drugs could be used as a potential and new treatment strategy for cochlear injury.

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