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PROSTAGLANDIN E RECEPTOR SUBTYPE EP4 AGONIST PROTECTS COCHLEAE AGAINST NOISE-INDUCED TRAUMA

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Abstract—Prostaglandin E_1 is frequently used for the clinical treatment of acute sensorineural hearing loss. However, the mechanisms underlying the effects of prostaglandin E_1 on the inner ear have not yet been elucidated. The physiological effects of prostaglandin E_1 are mediated by the prostanoid receptors prostaglandin I receptor and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4, the respective agonists for which have been purified. In the current study, we examined the efficacy of a local EP4 agonist application for the treatment of sensorineural hearing loss. We examined EP4 expression in the mouse cochlea using the reverse transcription–polymerase chain reaction and immunohistochemistry. The protective effects of local EP4 agonist treatment before or after noise exposure were tested in guinea pigs using measurements of auditory brain-stem responses and histological analysis. The results demonstrated EP4 expression in the cochlea, and showed that pre- and post-treatment with an EP4 agonist significantly attenuated threshold shifts of auditory brain stem responses, and significant attenuation in the loss of outer hair cells was found in local EP4 agonist treatment before noise exposure. These findings indicate that EP4 is involved in mechanisms for prostaglandin E_1 actions on the cochlea, and local EP4 agonist treatment could attenuate acute sensorineural hearing loss.

Key words: acoustic trauma, hair cell, hearing loss, inner ear, prostanoid.

Sensorineural hearing loss (SNHL) is one of the most frequent disabilities. Once hearing has been lost, it is rarely recovered, because the mammalian auditory system, particularly the sensory hair cells (HCs), has a limited capability for regeneration. Clinically, there are no curative therapeutic options for chronic SNHL, and the curative rate for acute SNHL is also limited. The systemic application of corticosteroids has been accepted as the primary treatment of choice for acute SNHL, although its efficacy has not been substantiated (Wei et al., 2006). In general, approximately 50% of SNHL cases show no response to the systemic application of corticosteroids (Ogawa et al., 2002). Other options for the treatment of acute SNHL have therefore been required. Prostaglandin E_1 (PGE_1) has often been used as a secondary choice treatment for acute SNHL. However, its clinical efficacy remains controversial (Ahn et al., 2005; Suzuki et al., 2008; Zhuo et al., 2008). PGE_1 is usually applied to improve local blood circulation. An experimental study using guinea pigs demonstrated an increased blood supply in the cochlea following local PGE_1 application (Tominaga et al., 2006). However, the actual mechanisms underlying the effects of PGE_1 on the inner ear have not yet been elucidated.

The physiological actions of PGE_1 are mediated by the prostanoid receptors prostaglandin I receptor (IP) and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4 (Coleman et al., 1994; Kiriyama et al., 1997). Recently, these prostanoid receptors have been cloned, and their actions have been elucidated. IP, EP2, and EP4 are coupled to G-protein stimulation and mediate increases in cyclic AMP (cAMP) that activate protein kinase A (PKA) (Coleman et al., 1994; Narumiya et al., 1999). The EP4 receptor in particular has various physiological and pathophysiological actions, including anti-apoptotic (Kataoka et al., 2005), anti-excitotoxicity (Ahmad et al., 2005), and anti-inflammatory (Kabashima et al., 2002; Nitta et al., 2002) effects. The actions of EP4 agonists could protect auditory HCs, and might indicate therapeutic efficacy of PGE_1 for acute SNHL. We therefore investigated the potential use of an EP4 agonist in the protection of auditory HCs from noise trauma. In the current study, we demonstrated EP4 expression in the cochlea using the reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry. We also showed the efficacy of local EP4 agonist treatment for protecting auditory HCs against noise-induced damage, by means of auditory brain-stem response (ABR) recordings and histological analyses of cochlear specimens.

EXPERIMENTAL PROCEDURES

Experimental animals

Male C57BL/6 mice at 8 weeks of age and Hartley guinea pigs weighing 350–400 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was supervised by

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Abbreviations: ABR, auditory brain-stem response; bp, base pairs; cAMP, cyclic AMP; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetra-acetic acid; EP, prostaglandin E receptor subtype; HC, hair cell; HGF, hepatocyte growth factor; IHC, inner hair cell; IP, prostaglandin I receptor; OHC, outer hair cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGE_1, prostaglandin E_1; PKA, protein kinase A; RT-PCR, reverse transcription–polymerase chain reaction; RWM, round window membrane; SNHL, sensorineural hearing loss; SPL, sound pressure level; VEGF, vascular endothelial growth factor.

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the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. We intended to minimize the numbers of animals and their suffering.

EP4 mRNA expression in mouse cochleae

Under general anesthesia with midazolam (10 mg/kg; Astellas, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Tokyo, Japan), mice were sacrificed and their cochleae were immediately collected. Each mouse cochlea was homogenized, and total RNA was extracted using an RNeasy mini kit (Qiagen Ltd., Valencia, CA, USA). Complementary DNA was synthesized from DNase I-treated total RNA using the Superscript first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed using a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The primers used in the PCR for EP4 and for β-actin as an invariant control were as follows: EP4 (424 base pairs [bp]), 5′-TTCCGCTCGTGGTGCGAGTGTTC-3′ (sense) and 5′-GAGGTGGTGTCTGCTTGGGTACG-3′ (antisense); and β-actin (321 bp), 5′-ACCAACTGGGACGACATGGAGAAGATCTGG-3′ (sense) and 5′-CCGGCCAGCCAGGTCCAGACGCAGGATGGC-3′ (antisense). The PCR conditions for EP4 were as follows: denaturation at 94 °C for 48 s, annealing at 64 °C for 42 s, and extension at 72 °C for 25 s. The PCR conditions for β-actin were as follows: denaturation at 94 °C for 48 s, annealing at 62 °C for 42 s, and extension at 72 °C for 72 s. The numbers of PCR cycles were 35 and 30 for EP4 and β-actin, respectively. All reactions were confirmed to be in the logarithmic phase by monitoring the PCR products obtained at the indicated number of cycles ($\pm$2). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR experiments were independently repeated three times using the same total RNA material. Extracts of mouse lungs were used as positive controls, and negative controls were obtained by omitting reverse transcriptase from the reactions.

Immunohistochemistry for EP4 in the cochlea

Immunohistochemistry was performed to examine the localization of EP4 in the auditory epithelia. Cochlear specimens from the mice were fixed with 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS; pH 7.4) at 4 °C for 12 h. After decalcification with 0.1 M EDTA for 7 days at 4 °C, 10-μm-thick cryostat sections were prepared. Anti-EP4 receptor polyclonal antibody (dilution, 1:200; Cayman Chemical, Ann Arbor, MI, USA) was used as the primary antibody for 12 h at 4 °C, and Alexa 568–conjugated goat anti-rabbit immunoglobulin G (dilution, 1:500; Molecular Probes, Eugene, OR, USA) was used as the secondary antibody for 1 h at room temperature. After immunostaining for EP4, the nuclei were counterstained with 4,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Heart specimens obtained from the mice were used as positive controls for EP4. Nonspecific labeling was tested by blocking protein–antibody complex formation using EP4 receptor blocking peptide (Cayman Chemical). The specimens were viewed with a Leica TCS-SPE confocal microscope (Leica Microsystems, Wetzlar, Germany).

Drug application and noise exposure

The EP4 agonist used was ONO-AE1-329 (Ono Pharmaceutical, Co., Ltd., Osaka, Japan). After the ABR measurements, the otic bulla of the left temporal bone of each guinea pig was exposed using a retroauricular approach under general anesthesia with midazolam (10 mg/kg i.m.) and xylazine (10 mg/kg i.m.). A small hole was made in the left bulla to expose the round window niche. A gelatin sponge in dry conditions was cut into pieces 1.5–2 mm^3 in size under microscopy. A piece of gelatin that had been immersed in the EP4 agonist, which had been dissolved in dimethyl sulfoxide (DMSO) and diluted with physiological saline to give a final concentration of 1 mg/ml containing 1% DMSO, was then placed on the round window membrane (RWM) of the animals in the EP4 agonist group (n=8). For the animals in the control group, a piece of gelatin that had been immersed in physiological saline containing 1% DMSO was used (n=9). The animals were exposed to one octave band noise centered on 4 kHz at a sound pressure level (SPL) of 120 dB for 5 h in a ventilated sound-exposure chamber immediately after drug application under general anesthesia with midazolam and xylazine. Each animal was immobilized, and a speaker was centered over the animal's head at a distance of 15 cm. The sound chamber was fitted with speakers driven by a noise generator and a power amplifier. Using a 1/2-inch condenser microphone (Sony, Tokyo, Japan) and fast Fourier transform analyzer (Sony), sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure uniformity of the stimulus. The stimulus intensity varied by a maximum of 3 dB SPL across measured sites within the exposure chamber. We also examined post-treatment effects. The animals were locally administered an EP4 agonist (n=5) or physiological saline containing 1% DMSO (n=5) 30 min after noise exposure. ABR thresholds following local application of saline containing 1% DMSO were measured in normal guinea pigs (n=4) as a control experiments.

ABR measurements

The ABR thresholds were measured at frequencies of 4, 8, and 16 kHz before noise exposure, and on days 3, 7, 14 and 21 after drug application. The animals were anesthetized with midazolam and xylazine, and kept warm with a heating pad. The generation of acoustic stimuli and the subsequent recording of evoked potentials were performed using a Powerlab/4sp (ADInstruments, Colorad Springs, CO, USA). The acoustic stimuli, which consisted of tone-burst stimuli (0.1 ms cos2 rise/fall and 1-ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected to a funnel fitted into the external auditory meatus. To record bioelectrical potentials, subdermal stainless-steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active), and contralateral to the measured ear (reference). The stimuli were calibrated against a 3-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). The thresholds were determined from a set of responses at varying intensities with 5-dB SPL intervals, and the electrical signals were averaged over 1024 repetitions. The thresholds at each frequency were verified at least twice.

Histological analyses

On day 21 after drug application, the temporal bones were collected and immersed in 4% PFA in 0.01 M PBS at 4 °C for 12 h. After decalcification with 0.1 M EDTA for 14 days at 4 °C, the cochleae were subjected to histological analysis as whole mounts. Three regions of the cochlear sensory epithelia, at a distance of 30%–50% (corresponding to 1–3 kHz regions, second turn), 50%–70% (corresponding to 3–8 kHz regions, mid-basal portion), and 70%–90% (corresponding to 8–30 kHz regions, basal portion) from the apex (Viberg and Canlon, 2004), were used for quantitative assessments of HC loss. Cochlear specimens were permeabilized in 0.2% Triton X in PBS for 30 min at room temperature. Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were used to label the inner hair cells (IHCs) and the outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (dilution, 1:500; Proteus BioSciences, Ramona, CA, USA)