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PRAVASTATIN ATTENUATES NOISE-INDUCED COCHLEAR INJURY IN MICE

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Abstract—Noise-induced hearing loss (NIHL) is one of the most common forms of sensorineural hearing loss and a well-known contributor to presbycusis. Based on the generation of reactive oxygen species (ROS) in the pathogenesis of NIHL, augmentation of the antioxidative defense system is a major target for pharmacological prevention. In this study, we assessed whether administration of pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which is a rate-limiting enzyme of cholesterol synthesis, before noise exposure protects against cochlear injury in BALB/c mice. Noise exposure produced both compound threshold shift (CTS) and permanent threshold shift (PTS) over 40 dB at 16 and 32 kHz. Pretreatment with pravastatin (25 mg/kg) for 5 days significantly decreased both CTS and PTS. Pravastatin also reduced hair cell death after noise exposure in the cochlea, which was identified by surface preparation and scanning electron microscopy (SEM). It also reduced the formation of noise-induced 4-hydroxynonenal (4-HNE), a byproduct of lipid peroxidation. Activation of Rac1, one of the subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is a major superoxide generator in the cell membrane, was inhibited by the administration of pravastatin. These findings suggest that pravastatin can protect against cochlear acoustic injury by lowering ROS generation via inhibition of the formation of the NADPH oxidase complex. This study will be helpful for the development of new therapeutic strategies for NIHL and other hearing loss-related diseases caused by ROS overproduction.

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Abbreviations: ABR, auditory brainstem response; CTS, compound threshold shift; DAB, 3, 3′-diaminobenzidine; EDTA, ethylenediaminetetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPD, hearing protection devices; NAC, *N*-acetyl cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NIHL, noise-induced hearing loss; OC, organ of Corti; OHC, outer hair cell; PB, phosphate buffer; PBS, phosphate buffered saline; PTS, permanent threshold shift; ROS, reactive oxygen species; SEM, scanning electron microscopy; 4-HNE, 4-hydroxynonenal.

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Noise-induced hearing loss (NIHL) is one of the most common forms of sensorineural hearing loss and one of the most common occupational diseases in both developed and developing countries. Approximately, 30 million workers are exposed to hazardous noise. According to the National Institute of Occupational Safety and Health and Veterans Affairs, compensation rates for NIHL are over 1 billion dollars per year in the United States (Fausti et al., 2005). Acoustic overexposure also has been proposed as a contributor to presbycusis because of the pathophysiological similarities between the two diseases (Ohlemiller, 2008). Currently, the most efficient method to prevent NIHL is wearing hearing protection devices (HPD). But, there can be inadequate protection and noncompliance with HPD because wearing HPD causes discomfort and head movement restriction and impairs the ability to communicate. In high-noise environments, alternative approaches should be considered. The protective effect of HPD could be augmented by the addition of pharmacological agents for noise protection (Oishi and Schacht, 2011).

Researchers have defined several pathophysiological mechanisms involved in NIHL, including glutamate excitotoxicity, ischemia/reperfusion injury derived from the stria vascularis, ion imbalance in the endolymph, and oxidative stress (Henderson et al., 2006). Among them, recent clinical and experimental studies have focused on the generation of reactive oxygen species (ROS) in the pathogenesis of NIHL (Henderson et al., 2006). The main source of ROS in the inner ear is the mitochondrial electron transport chain in NIHL (Henderson et al., 2006). Therefore, noise metabolically stresses hair cells, which causes hair cell death. Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is also suggested to generate ROS from excessive noise (Ramkumar et al., 2004). Accordingly, there are many antioxidant systems that neutralize ROS in the inner ear, and these antioxidant systems include catalase, glutathione reductase, and γ-glutamylcysteine synthetase, which are increased by noise (Henderson et al., 1999). However, such an unwanted byproduct overwhelms the natural antioxidative defense systems, which causes hearing loss. Thus, great efforts have been made to boost up these antioxidant systems in the inner ear through administration of antioxidant agents such as *N*-acetyl cysteine (NAC), lipoic acid, resveratrol, and superoxide dismutase-polyethylene glycol (Lynch and Kil, 2005; Oishi and Schacht, 2011). Early studies used NAC, a well-known ROS scavenger, to attenuate the ROS toxicity (Kopke et al., 2000, 2007; Duan et al., 2004; Lin et al.,

2010). But no consistent results were reported by the use of NAC for NIHL (Kramer et al., 2006; Hamernik et al., 2008; Davis et al., 2010). In addition, the efficacy of known antioxidants may be limited by several factors, including limited access to cellular compartments and action against only a few forms of ROS (Le Prell et al., 2007). Therefore, other antioxidants or combinations of multiple antioxidants with different mechanisms are needed to be developed.

Statins are commonly used as cholesterol-lowering drugs and have been shown to reduce the incidence of primary and secondary coronary heart disease (Nassief and Marsh, 2008). The primary target of statins is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibition, which results in reduction of cholesterol synthesis, mainly in the liver. In other organs, it can deplete membrane cholesterol and affect membrane associated cellular signaling. In addition to their cholesterol-lowering effects, recent studies show that statins possess many other pharmacologic effects such as anti-inflammatory, antioxidative, and neuroprotective effects independent of their effects on lipid metabolism (Blum and Shamburek, 2009). These pleiotropic effects of statins are proposed to be derived from their inhibition of the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which serve as important lipid moieties for the posttranslational modification of a variety of proteins, including the small GTP-binding proteins, Ras, Rho, and Rac GTPase (Rikitake and Liao, 2005). In particular, Rac1, one of the small GTPase superfamily proteins, is involved in superoxide generation via NADPH oxidase complex formation, and it is proposed that statin inhibits Rac1-mediated activation of NADPH oxidase in smooth muscle and heart through inhibition of isoprenylation as a possible mechanism of their antioxidative effects (Wang et al., 2008). This has led to efforts applying them to new indications such as inflammatory, oxidative stress-related diseases, and neurodegenerative diseases (Davignon and Leiter, 2005).

In an effort to search for new safe drugs against NIHL, we explored the preventive effect of pravastatin on NIHL and the possible mechanisms of protecting the cochlea from acoustic overexposure in a BALB/c mice model system.

EXPERIMENTAL PROCEDURES

Experimental groups

The protocol used in this study was approved by the animal protocols and guidelines established by the Ajou University School of Medicine Ethics Review Committee for Animal Experiments, and all animal work was approved by the Ethical Committee for Animal Research of Ajou University (AMC-55). All efforts were made to minimize the number of animals used and their suffering. This study used 7–8-week-old BALB/c male mice (Orient Bio, Seoul, Korea). Animals were examined immediately or within 24 h after noise exposure and were maintained for 3 or 7 days for histological evaluation or for 14 days to evaluate permanent threshold shift (PTS; Fig. 1). The statin pretreatment group was pretreated with 25 mg/kg of pravastatin (Daiichi Sankyo, Tokyo, Japan) orally via gavage every midnight for 5 days. The noise-only group was treated with saline gavage at the same time. The control animals were neither exposed to noise nor given pravastatin.

Noise exposure

Exposure to noise was performed in a sound chamber. The ventilated chamber was fitted with a high frequency driver (Electro-Voice DH-7, Burnsville, MN, USA) driven by a function/arbitrary waveform generator (Agilent 33210 A, Santa Clara, CA, USA), which generates a 0.1 Hz–15 MHz range sound wave and power amplifier (AX5505, Inkel, Korea). The driver was located directly above the animals. Animals were exposed to a 112-dB sound pressure level (dB SPL) of broad-band white noise (1–20 kHz) measured by a dual channel real time frequency analyzer (type 2144, Brüel and Kjær, Nærum, Denmark) for 3 h. Each animal was separated by a wire net cage with a diameter of 10 cm to abolish the attenuation of sound pressure by adjacent animals.

Measurement of serum cholesterol content

Quantification of total cholesterol (measuring both cholesterol and cholesteryl ester) was performed using the Cholesterol/Cholesteryl Ester Quantification Kit (Invitrogen, Carlsbad, CA, USA), which is a colorimetric method. Briefly, the facial vein was punctured to obtain venous blood, and serum was collected after centrifugation, followed by analysis according to the manufacturer's protocol.

Auditory Brainstem Response analysis

Auditory Brainstem Response (ABR analysis was used to measure auditory thresholds in all subjects. ABR measurements were

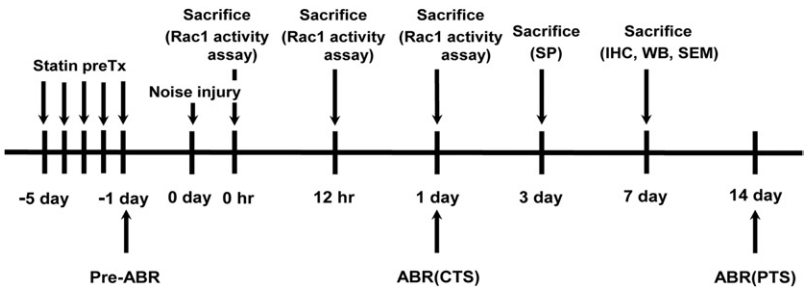

Fig. 1. Experimental scheme for the *in vivo* NIHL mouse model with pravastatin pretreatment. The statin pretreatment group was pretreated with 25 mg/kg of pravastatin via gavage every midnight for 5 d. An initial ABR test was performed on all animals to rule out innate hearing impairment and to check baseline hearing thresholds 1 d before noise exposure. At day 0, 112 dB of SPL white noise was given for 3 h. Immediately after noise exposure, mice were sacrificed for Rac1 activity at 12-h intervals until day 1. At day 1, a CTS measurement was performed on all noise-exposed animals. At days 3 and 7, animals were sacrificed for surface preparation (SP), immunostaining, Western blot (WB), and scanning electron microscopy (SEM). At day 14, a PTS measurement was performed on the remaining animals.