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Inducible Nitric Oxide Synthase Participates in Cochlear Damage after Acoustic Stimulation in Guinea Pigs

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Abstract

Inducible nitric oxide synthase (iNOS/NOS II) mediates cytotoxicity under pathological stimulation. The purpose of this study was to examine whether the blockade of NOS activity leads to a decrease in cochlear damage after intense acoustic stimulation. Guinea pigs were divided into 4 groups: (1) a noise group, (2) a NOS inhibitor (N^G-nitro-L-arginine methyl ester [L-NAME]) + noise group (L-NAME/noise group), (3) an L-NAME group, and (4) a control group. Stimuli involved a pure tone at a frequency of 2 kHz for 5 hours. The sound pressure level was 120 dBSPL. In the L-NAME/noise group, 50 mg/kg body weight of L-NAME was injected 1 hour before acoustic stimulation. In the control group and the L-NAME group, acoustic stimulation was not performed. In the L-NAME group, the same dose of L-NAME was injected intraperitoneally. In the control group, only physiological saline was injected. Auditory brainstem responses (ABRs) were recorded before and immediately, 1 day, and 7 days after acoustic stimulation. The ABR threshold was significantly higher immediately after acoustic stimulation in both the noise group and the L-NAME/noise group. One day after acoustic stimulation, the threshold shift was decreased in the noise group. The threshold shift was still present 7 days after acoustic stimulation but was significantly lower in the L-NAME/noise group than in the noise group. In the L-NAME group and the control group, threshold shifts were not apparent. The lateral wall, the organ of Corti, and the spiral ganglion cells of the cochlea in both the L-NAME group and the control group did not display immunoreactivity for iNOS at any time. Immunoreactivity for iNOS was found in the lateral wall, the supporting cells (Hensen's cells, Deiters' cells, and pillar cells), and the spiral ganglion cells in both the noise group and the L-NAME/noise group. These immunoreactivities for iNOS were detected immediately, 1 day, and 7 days after acoustic stimulation. Immunoreactivity decreased over time in the stria vascularis, the organ of Corti, and the spiral ganglion cells in the noise group. The same phenomenon was observed in the L-NAME/noise group. In conclusion, iNOS was detected in cochlea damaged by acoustic stimulation. A NOS inhibitor (L-NAME) reduced the elevation of hearing thresholds. Our results suggest that the expression of iNOS participates in the pathogenesis of cochlear damage caused by acoustic trauma.
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Key words: nitric oxide, inducible nitric oxide synthase, acoustic trauma, auditory brainstem response

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Introduction

Nitric oxide (NO) is synthesized from L-arginine in mammals by the action of NO synthase (NOS). Constitutive NOS, such as endothelial NOS (eNOS/NOS III) and brain NOS (bNOS/NOS I), plays physiological roles in humans. On the other hand, it has become clear that inducible NOS (iNOS/NOS II), which is an isoform of NOS, mediates cytotoxicity under pathological stimulation[1–3]. iNOS produces large amounts of NO and reacts with superoxides, which are harmful to surrounding tissues. Various pathological conditions, such as inflammation[4–6] and endolymphatic hydrops[7–9], and pharmaceutical agents, such as anticancer drugs[10,11] and gentamicin[12], promote the expression of iNOS.

Acoustic trauma is commonly encountered at ear, nose, and throat clinics[13]. Intense noise, such as that at a rock concert or from a gunshot, causes inner ear damage, such as hearing disturbance, decreased speech discrimination, and recruit phenomenon[13]. We have reported that acoustic stimulation promotes the expression of iNOS in the vestibule of guinea pigs[14]. The purpose of the present study was to examine whether the blockade of NOS activity leads to a decrease in cochlear damage after intense acoustic stimulation.

Materials and Methods

Forty guinea pigs weighing 250 to 350 g were used. All animals were confirmed to have a Preyer's reflex. The animals were anesthetized adequately with 5% (w/v) ketamine hydrochloride (50 mg/kg body weight) before all procedures. The animals were divided into 4 groups: (1) a noise group (n=10), (2) a NOS inhibitor (N^G-nitro-L-arginine methyl ester [L-NAME]) + noise group (L-NAME/noise group) (n=10), (3) an L-NAME group (n=10), and (4) a control group (NaCl 0.9% w/v) (n=10). A tissue specimen from a patient with squamous cell carcinoma of the epipharynx was used as a positive control. The control group was used as the negative control. The protocol used was in accordance with the guidelines for research involving animals and was approved by the ethics committee of our institution (No. 12–84, 13–30, 15–103 and 19–107).

Acoustic Stimulation

The head of each animal was fixed; however, animals were adequately fed during acoustic stimulation. Acoustic stimuli were applied for 5 hours in a soundproof room using a sound synthesizer (Wave Factory WF1943, FF Kairo-sekkei Block Co., Kanagawa), an amplifier (Dual Power Amplifier IP600D, FF Kairo-sekkei Block Co.), and a loudspeaker (Horn Drivers D1400, Fostex, Tokyo). The loudspeaker was placed beside the right ear. Stimuli were pure tone, and the frequency was 2 kHz. The sound pressure level was 120 dBSPL. In the control group and the L-NAME group, acoustic stimulation was not performed.

NOS-inhibitor (L-NAME) Injection

In the L-NAME/noise group, 50 mg/kg body weight of L-NAME (5 mg/mL; Lot. No. 37H0382, Sigma-Aldrich, St. Louis, MO, USA) dissolved in physiological saline (NaCl 0.9% w/v) was injected intraperitoneally 1 hour before acoustic stimulation. In the L-NAME group, the same dose of L-NAME was injected. In the control group, only physiological saline (10 mL/kg, NaCl 0.9% w/v) was injected intraperitoneally.

Auditory Brainstem Response Measurement

Auditory brainstem response (ABR) recordings were made with an electrodiagnostic system (Kissei Comtec. Co. Ltd., Tokyo) before and immediately, 1 day, and 7 days after acoustic stimulation. The active electrode was inserted subcutaneously into the ipsilateral pinna, the reference electrode into the contralateral pinna, and the ground electrode into the top of the head. Acoustic stimuli were delivered with an earphone through a small tube inserted into the external auditory meatus in a soundproof box. The stimuli consisted of clicks that were presented at a rate of 11.1 per second and a duration of 0.11 milliseconds. Responses were accumulated 200 times. The levels of stimuli were reduced from 103 dBSPL to 33 dBSPL in 5-dB steps. The ABR threshold was determined as the minimum sound level giving

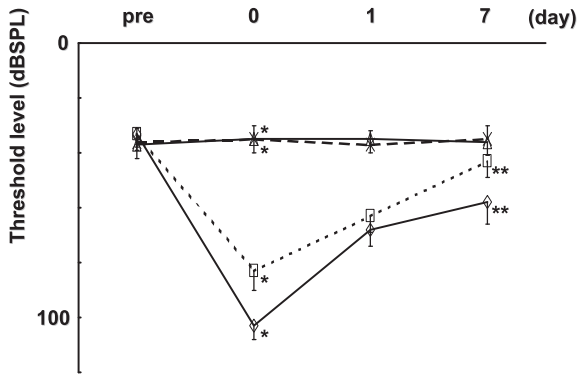

Fig. 1 The means ± SD of the threshold shift of ABR
The ABR threshold level was significantly elevated immediately after acoustic stimulation in both the group 1 (solid line with diamond) and the group 2 (short broken line with square) (ANOVA, $p<0.01$*). One day after acoustic stimulation, the threshold shift was decreased in the group 1. The threshold shift was still present 7 days after acoustic stimulation; however, the threshold shift was significantly lower in the group 2 than the group 1 (ANOVA, $p<0.05$**). In the group 3 (solid line with triangle) and the group 4 (long broken line with cross mark), threshold shifts were not apparent.

reproducible waveforms.

Immunohistochemical Examination

Two animals in each group were sacrificed immediately and 1 day after acoustic stimulation for histochemical study. The remaining 6 animals in each group were also used for histochemical study. The tissues were fixed through cardiac perfusion with 4% (w/v) paraformaldehyde. The temporal bones were immersed in the same fixative overnight. The specimens were embedded in paraffin after decalcification by incubation in a solution of 10% EDTA for 5 days. Each specimen was cut into 8-μm-thick section with a microtome (Leica, Bartles and Stout, Issaquah, WA, USA). After the paraffin was removed, the sections were immersed in 3% H_2O_2 for 30 minutes and then in Triton X for 10 minutes. Subsequently, the sections were incubated with the primary antibody to iNOS at 1 : 2,000 dilution (rabbit polyclonal antibody, SA-200, Lot. No. P.8557, Biomol/Enzo Life Science, Farmingdale, NY, USA) overnight. After being rinsed with Tris buffer solution and normal goat serum, the tissues were incubated with the second antibody at 1 : 400 dilution (anti-rabbit, Dako, Glostrup, Denmark). Development was performed with horseradish peroxidase at 1 : 100 dilution for 1 hour and nickel-enhanced diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka).

Results

Threshold Shifts of ABR

The threshold shifts of the ABR before and immediately, 1 day, and 7 days after acoustic stimulation are shown in **Figure 1**. The threshold level of the ABR was elevated significantly immediately after acoustic stimulation in both the noise group and the L-NAME/noise groups (analysis of variance [ANOVA], $p<0.01$*). One day after acoustic stimulation, the threshold shift was decreased in the noise group. The threshold shift was still present 7 days after acoustic stimulation; however, the threshold shift was significantly lower in the L-NAME/noise group than in the noise group (ANOVA, $p<0.05$**). In the L-NAME group and control group, threshold shifts were not apparent.

Immunohistochemical Expression of iNOS

The cytoplasm of squamous cell carcinoma of the epipharynx exhibited immunostaining for iNOS (**Fig. 2a**). The lateral wall, the organ of Corti, and the spiral ganglion cells of the cochlea in the control group did not show immunoreactivity for iNOS at any time (**Fig. 2b–e**). In the L-NAME group, iNOS reactivity was not also observed.

Immunoreactivity for iNOS was found in the lateral wall, the supporting cells (Hensen's cells, Deiters' cells, and pillar cells), and the spiral ganglion cells in both the noise group (**Fig. 3**) and the L-NAME/noise group. These immunoreactivities to iNOS were detected immediately, 1 day, and 7 days after acoustic stimulation.

Immunoreactivity in the stria vascularis, the organ of Corti, and the spiral ganglion cells decreased over time in the noise group (**Fig. 4**). The same phenomenon was observed in the L-NAME/noise

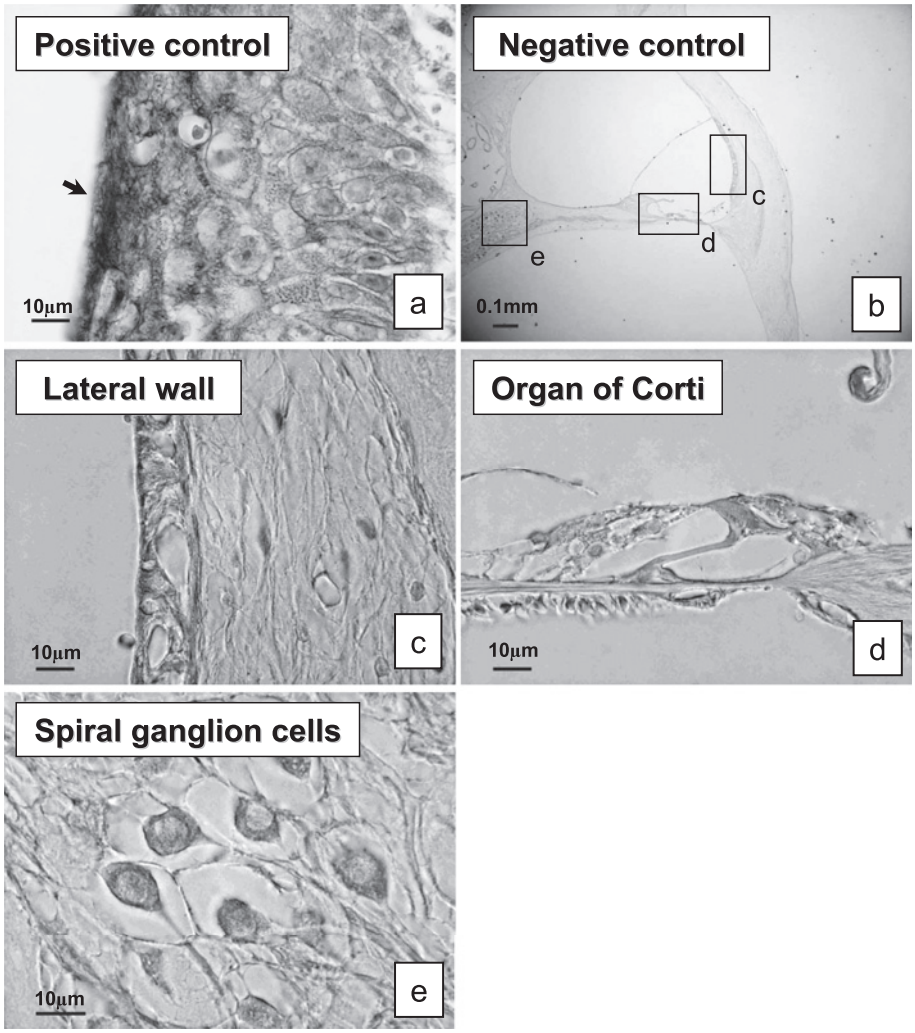

Fig. 2 Paraffin sections of the cochlea, 8-μm-thick. Immunohistochemistry, anti-iNOS.

a: Positive control
The cytoplasm of squamous cell carcinoma of the epipharynx exhibited immunostaining for iNOS (**arrow**). ×150.

b: Negative control
The cochlea in the control group 5 hours after acoustic stimulation is shown. The structure of the cochlea did not exhibit immunoreactivity for iNOS. ×10.

c: Lateral wall of cochlea in the control group
The stria vascularis did not show immunoreactivity for iNOS. ×150.

d: Organ of Corti in the control group
The organ of Corti did not show immunoreactivity for iNOS. ×150.

e: Spiral ganglion cells in the control group
iNOS reactivity was not observed in the cytoplasm of spiral ganglion cells. ×150.

group. However, the immunoreactivity was weaker in the L-NAME/noise group than in the noise group.

Discussion

In the present study, we detected iNOS expression in the lateral wall, the organ of Corti, and the spiral ganglion cells of guinea pigs exposed to intense acoustic stimulation. We have previously reported that iNOS is expressed in various pathological conditions of the inner ear, such as endolymphatic hydrops[8] and acute inflammation[6], and with the administration of cisplatin, an anticancer drug[10,11]. Under these conditions, iNOS

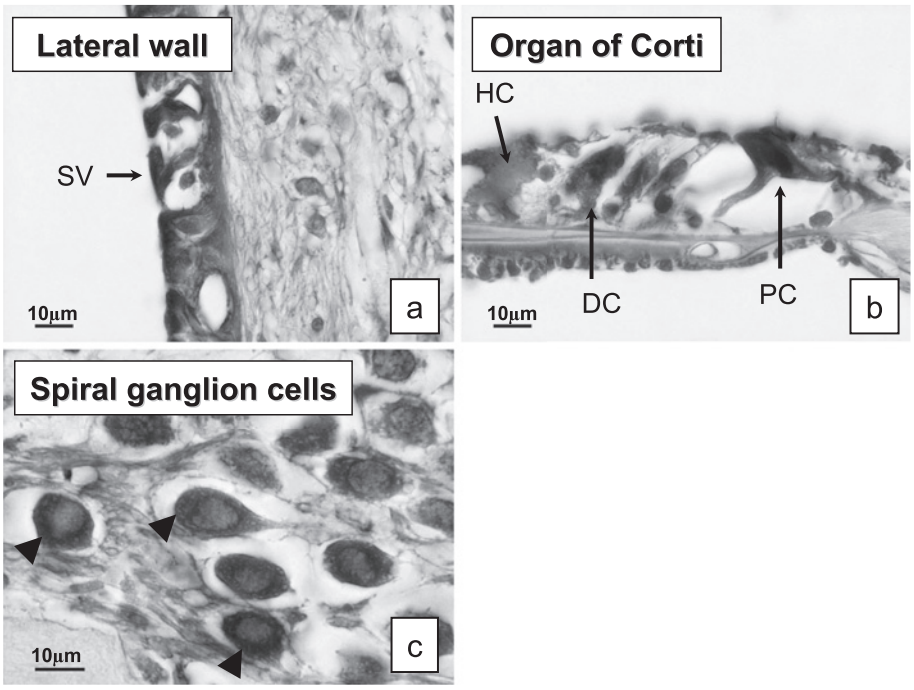

Fig. 3 Paraffin-embedded sections of the cochlea, 8-μm-thick. Immunohistochemistry, anti-iNOS, in the noise group 5 hours after acoustic stimulation.
a: Lateral wall of cochlea
The stria vascularis (SV) showed intense immunoreactivity for iNOS. ×150.
b: Organ of Corti
Supporting cells; Hensen's cells (HC), Deiters' cells (DC), and pillar cells (PC) showed immunoreactivity for iNOS. ×150.
c: Spiral ganglion cells
The cytoplasm of spiral ganglion cells showed immunoreactivity for iNOS **(arrowheads)**. ×150.

catalyzes large amounts of NO, a free radical, and reacts with superoxide. The surrounding tissues are then damaged.

Acoustic stimulation is applied to the inner ear via the tympanic membrane, ossicles, and oval window. Under physiological conditions, the mechanical stimulation, produced by the sliding of the tectorial membrane over outer hair cells, is converted to electrical stimulation. On the other hand, loud acoustic stimulation damages the inner ear because the stress involved is greater than that in normal physiological stimulation[15,16]. This stress causes physical damage and a disorder of energy metabolism[17]. The lateral wall, especially the stria vascularis, plays a role in ion transport to maintain homeostasis of the inner ear. When the lateral wall is damaged, the energy metabolism of the inner ear is reduced, and a disturbance of the inner ear occurs. The supporting cells in the organ of Corti play a role in maintaining the structure of hair cells. The expression of iNOS in the organ of Corti indicates that the framework has been destroyed and that the mechanical stimulation produced by the sliding of the tectorial membrane over outer hair cells is not effectively being converted to electrical stimulation. Both the inner and outer hair cells are believed to be destroyed directly by damage to supporting cells and indirectly by the loss of energy metabolism. NO mediates the neurotoxicity of glutamate through the activation of excitatory amino acid receptors, especially N-methyl-D-aspartate (NMDA) receptors[18–20]. NMDA receptors have been detected in the cochlea[21]. Large amounts of NO increase the negative feedback mechanism on the NMDA receptors and might lead to apoptosis in the spiral ganglion cells. Thus, inner ear damage by

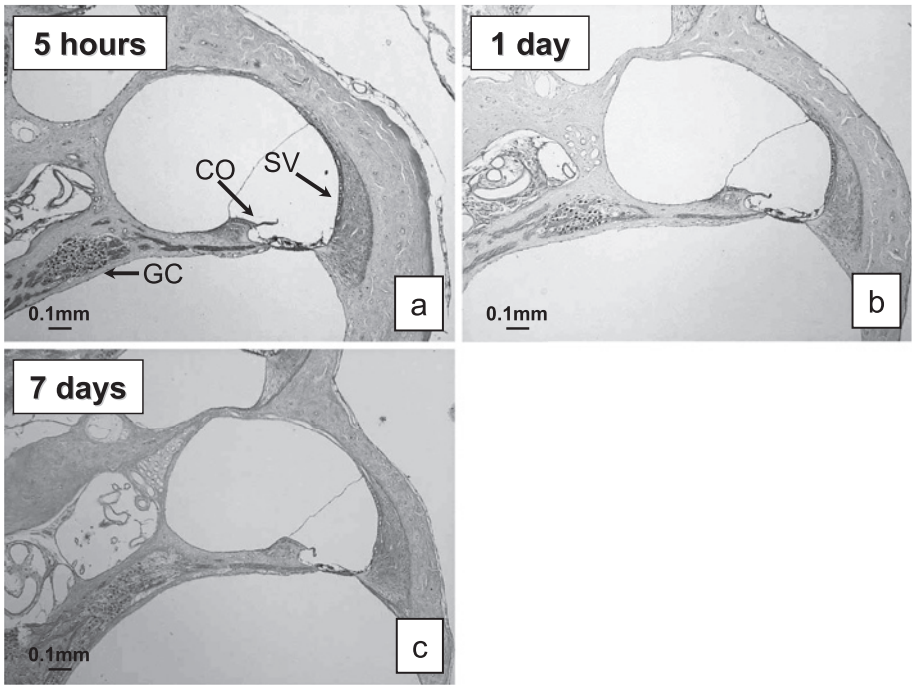

Fig. 4 Time-course of changes in expression of iNOS in the noise group

a: 5 hours after stimulation
Immunoreactivity for iNOS was found in the stria vascularis (SV), the organ of Corti (CO), and the spiral ganglion cells (GC) in the noise group. ×10.

b: 1 day
Immunoreactivity for iNOS was decreased. ×10.

c: 7 days
Immunoreactivity for iNOS was not apparent. ×10.

acoustic trauma is mediated in part by the iNOS-catalyzed NO pathway.

The degree of inner ear damage after acoustic stimulation depends on the sound pressure level, the duration, and the frequency. There are 2 mechanisms of noise injury, namely, temporary threshold shift (TTS) and permanent threshold shift (PTS)[22]. TTS is produced by short-term noise stress, is reversible, and is a phenomenon of auditory fatigue. In contrast, PTS is a permanent morphological change of the inner ear which is produced by the repetition of noise stress without the recovery of TTS. In the present study, we found that the threshold level of ABRs was elevated immediately after acoustic stimulation in the noise group and the L-NAME/noise group. We believe these phenomena are due to TTS. One day after acoustic stimulation, the threshold shift was improved. After 7 days, the threshold shift had decreased but was still present. We believe that PTS was exhibited in the noise group.

We also found that the threshold shift and the immunoreactivity to iNOS was less in the L-NAME/noise group than in the noise group 7 days after acoustic stimulation. L-NAME is an L-arginine analogue; it is a competitive inhibitor of NOS and nonspecifically inhibits both constitutive NOS (cNOS) and iNOS activity. The amount of NO produced by iNOS is 100 to 1,000 times that by cNOS. We observed no hearing threshold shift in the L-NAME group. This finding indicates that cNOS has little effect on inner ear damage after acoustic stimulation. These high levels of NO and free radical species thus seem responsible for the cytotoxic effect[3,18]. The decreased expression of iNOS in the L-NAME/noise group explains why L-NAME decreased the activity of iNOS. However, the immunoreactivity for iNOS indicates that iNOS is present but not in an amount equal to NO. To compensate for the limitations of the immunohistochemical method, we also performed electrophysiological analysis. We believe that under

pathological conditions, L-NAME acts on the iNOS pathway and leads to the suppression of cochlear damage.

In the present study, we demonstrated the presence of iNOS in the cochlea after 5 hours of loud acoustic stimulation. A sound pressure level greater than 110 dBSPL is necessary to produce a model of acoustic trauma[13–15]. An 8-kHz noise at 103 dBSPL produces a PTS[22]. The maximum sound pressure level of our loudspeaker was 120 dBSPL; therefore, we set the sound pressure level at 120 dBSPL. Concerning the duration of the acoustic simulation, we set periods of 1 hour, 2 hours, 5 hours, and 20 hours (unpublished observations). We found that a threshold shift for acoustic stimuli of 5 hours and 20 hours induced irreversible inner ear damage. Thus, we selected the stimulation time of 5 hours to ensure cochlear damage.

In patients with acoustic trauma, such as hearing loss due to loud rock music, dance music, or a gunshot, we have used steroids, vitamins, and other drugs to increase blood circulation. Such treatments are not always effective. In the present study, a NOS inhibitor clearly reduced inner ear damage due to intense noise. Therefore, our result suggests new possibilities for therapy and will be beneficial for patients with acoustic trauma.

In conclusion, iNOS was detected in cochlea damaged by acoustic stimulation. A NOS inhibitor (L-NAME) reduced the elevation of hearing thresholds. Our results suggest that expression of iNOS participates in the production of cochlear damage caused by acoustic trauma.

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References

1. Palmer RM, Ferrige AG, Moncada S: Nitric Oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524–526.
2. Bredt DS, Snyder SH: Nitric oxide, a novel neuronal messenger. Neuron 1992; 8: 3–11.
3. Huie RE, Padmaja S: The reaction of NO with superoxide. Free Rad Res Commun 1993; 18: 195–199.
4. Takumida M, Anniko M: Lipopolysaccharide-induced expression of nitric oxide synthase II in the guinea pig vestibular end organ. Eur Arch Otorhinolaryngol 1998; 255: 184–188.
5. Hess A, Bloch W, Huverstuhl J, et al.: Expression of inducible nitric oxide synthase (iNOS/NOS II) in the cochlear of guinea pigs after intratympanical endotoxin-treatment. Brain Res 1999; 830: 113–122.
6. Watanabe K, Hess A, Zumegen C, et al.: Changes of the compound action potential (CAP) and the expression of inducible nitric oxide synthase (iNOS/NOS II) in the cochlea under the inflammatory condition. Hear Res 2000; 145: 149–155.
7. Hess A, Bloch W, Su J, Stennert E, Addicks K, Michel O: Expression of inducible nitric oxide-synthase in the vestibular system of hydropic guinea pigs. Neurosci Lett 1999; 264: 145–148.
8. Watanabe K, Tomiyama S, Jinnouchi K, Pawankar R, Yagi T: Expression of inducible nitric oxide synthase in the cochlea following immune response in the endolymphatic sac of guinea pigs. ORL 2001; 63: 155–159.
9. Watanabe K, Tomiyama S, Jinnouchi K, Hess A, Michel O, Yagi T: Expression of inducible nitric oxide synthase (iNOS/NOS II) in the hydropic vestibule after injection of keyhole limpet hemocyanin into the endolymphatic sac of guinea pigs. Vestib Res 2001; 11: 67–71.
10. Watanabe K, Hess A, Bloch W, Michel O: Nitric Oxide synthase inhibitor suppresses the ototoxic side effect of cisplatin in guinea pigs. Anti-cancer Drugs 2000; 11: 401–406.
11. Watanabe K, Hess A, Michel O, Yagi T: Nitric oxide synthase inhibitor reduces the apoptotic change in the cisplatin-treated cochlea of guinea pigs. Anti-cancer Drugs 2000; 11: 731–735.
12. Heinrich UR, Selivanova O, Brieger J, Mann WJ: Endothelial nitric oxide synthase upregulation in the cochlea of the guinea pig after intratympanic gentamicin injection. Eur Arch Otorhinolaryngol 2006; 263: 62–68.
13. Borg E, Canlon B, Engström B: Noise-induced hearing loss. Scand Audiol Suppl 1995; 40: 1–147.
14. Watanabe K, Inai S, Hess A, Michel O, Yagi T: Acoustic stimulation promotes the expression of inducible nitric oxide synthase in the vestibule of guinea pigs. Acta Otolaryngol Suppl 2004; 553: 54–57.
15. Kopke RD, Coleman JK, Liu J, Campbell KC, Riffenburgh RH: Candidate's thesis: enhancing intrinsic cochlear stress defenses to reduce noise-induced hearing loss. Laryngoscope 2002; 112: 1515–1532.
16. Nishimoto K, Nakagawa C, Wada H, Tsuchiya K, Sawaki S: Experimental studies on fatigue of inner ear by cochlear microphonics. Jibi-to-rinsyou 1988; 34: 1014–1021.
17. Hu BH, Henderson D, Nicotera TM: Extremely rapid induction of outer hair cell apoptosis in the chinchilla cochlea following exposure to impulse noise. Hear Res 2006; 211: 16–25.
18. Cazevieille C, Muller A, Meynier F, Bonne C: Superoxide and nitric oxide cooperation in hypoxia/reoxygeneration-induced neuron injury. Free Rad Biol Med 1993; 14: 389–395.

19. Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH: Nitric oxcide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci USA 1991; 88: 6368–6371.
20. Dawson VL, Dawson TM, Bartley DA, Uhl GR, Snyder SH: Mechanisms of nitric oxcide-mediated neurotoxicity in primary brain cultures. J Neurosci 1993; 13: 2651–2661.
21. Puel JL, Ladrech S, Chabert R, Pujol R, Eybalin M: Electrophysiological evidence for the presence of NMDA receptors in the guinea pig cochlea. Hear Res 1991; 51: 255–264.
22. Buck K, Franke R: Can TTS be an indicator for individual susceptibility to PTS? In Basic and applied aspects of noise-induced hearing loss: Nato Sci Series A: VOL III (Salvi RJ, Henderson D, Hamernik RP, Colletti V, eds), 1986; pp 441–456, Plenum Press, New York.

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