Abstract. Significant advances have been made in our understanding of the functional significance of nitric oxide (NO) in the inner ear. The localization of NO synthase and the nitric oxide production site has now been established by immunohistochemistry and the fluorescent indicator of NO. The functional significance of NO in the inner ear, especially as a neurotransmitter, is becoming increasingly clear. Mounting evidence suggests that excessive NO production may play an essential role in inner ear disorders as well. The production of an inducible type of NO synthase may be closely related to this phenomenon. Based on the mechanisms of inner ear disorders, new pharmacological strategies for preventing and/or treating inner ear disorders have also been suggested.

Controlled production of nitric oxide (NO) plays an important role in mediating neurotransmission, regulating vascular tone and in pathophysiology. NO is synthesized by NO synthase (NOS) in an unusual reaction that converts arginine (Arg) and oxygen into citrulline and NO. Although several NOS isoforms have been isolated, all are homologous and fall into two categories with different activities and regulatory mechanisms. The constitutive neuronal (NOS I, nNOS, ncNOS) and endothelial (NOS III, eNOS, ecNOS) NOS are always present. These NOS isoforms are inactive until intracellular calcium levels increase, the calcium-binding protein calmodulin binds to calcium and the calcium-calmodulin complex binds to and activates NOS. The constitutive types of NOS (cNOS) synthesize small amounts of NO until calcium levels decrease. This intermittent production of small amounts of NO transmits signals.

In contrast, the inducible NOS isoform, NOS II (iNOS), is usually absent, but can be synthesized by virtually any cell type when adequately stimulated with cytokines or some other agent. Once produced, it invariably synthesizes large amounts of NO. The continuous production of a large quantity of NO kills or inhibits pathogens.

Recent evidence suggests that NO may play a significant role in the inner ear. Recent years has seen important advances in our understanding of the action of NO in the inner ear, not only in normal but also under pathological conditions, which this review summarizes and evaluates.

Functional significance of NO in the inner ear

Constitutive isoforms of NOS (NOS I and III). In the inner ear, constitutive isoforms of NOS (NOS I and III) have recently been identified both in the cochlea and in the vestibular end organs, mainly by using NADPH-diaphorase histochemistry and immunohistochemistry (1-5). In the organ of Corti, NOS I was localized in the nerve fibres of the spiral ganglion itself, spiral ganglion cells, the nerve fibres, nerve endings below the inner and outer hair cells, the inner and outer hair cells, Deiters' cells, pillar cells, stria vascularis and spiral ligament (1,2,4,5). In the vestibular end organs, i.e. crista ampullares, utricular and saccular maculae, NOS I was localized in the vestibular ganglion cells, nerve fibres, the cytoplasm of both type I and type II sensory cells, the nerve fibres contacting the type II cells, dark cells and transitional cells (2, 3).

In the cochlea, NOS III was localized in the nerve fibres of the spiral ganglion, the spiral ganglion cells, the nerve fibres, nerve endings below the inner and outer hair cells, the inner and outer hair cells, Deiters' cells, pillar cells, stria vascularis, spiral ligament, the endothelium of blood vessels.
below the basilar membrane, the surrounding small capillaries in the lateral wall tissue and strial capillaries (1, 2, 4, 5). In the vestibular end organs, NOS III was localized in the vestibular ganglion cells, nerve fibres, the cytoplasm of both type I and type II sensory cells, the nerve fibres contacting the type II cells, dark cells, transitional cells and blood vessels in the subepithelial tissues (2,3).

As in many other organ systems, NO also plays an important role in the inner ear to regulate physiological reactions in both the cochlea and vestibular parts of the labyrinth. With regard to the effects of NO on neurotransmission, there is general consensus that NOS inhibitors may inhibit (and NO donors may facilitate) the electrical activity of afferent neurons. NO significantly contributes to the basal discharge and to the response of afferent fibres to mechanical stimuli. NO from the postsynaptic nuclei binds to the N-methyl-D-aspartic acid (NMDA) receptor. NO acts directly on a side of the receptor channel complex such as the redox modulatory side. NO binding on the redox modulatory side reduces the activity of the NMDA receptor (6). In the organ of Corti, a negative feedback mechanism has been suggested. Excitation of inner hair cells causes liberation of glutamate, which stimulates NMDA-type receptors on the afferent synaptic terminal. In addition, stimulation of the NMDA receptor liberates NO, which diffuses out of the synaptic terminal and inhibits the NMDA receptor (negative feedback mechanism). This hypothesis is supported by the finding that iontophoretically applied L-N(5)-nitroarginine methylester (L-NAME), a potent NOS inhibitor, augmented the NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-induced increase in afferent fibre activity (1, 7).

NO produced in an efferent button could increase synaptic transmission in the hair cells contacted by the button, but NO produced in a hair cell could only increase transmission in neighbouring hair cells and not in the same hair cell (8). NO-producing efferents release NO in the periphery. This in turn would stimulate the afferents into which they feed. Thus efferents acting on hair cells in the periphery would produce a type of positive feedback on both the afferents and the hair cells.

Compelling physiological evidence supports a role for the inner ear NO in the regulation of cochlear and vestibular blood flow. Round window membrane application of NO donors increases cochlear and vestibular blood flow (9). Conversely, the NOS inhibitor L-NAME reduces cochlear blood flow (10). The observed decrease of cochlear blood flow was presumably the result of a decrease in NO production, which was confirmed in the current study of isolated cochlear vascular endothelium (11). These data strongly suggest that NO production from cochlear and vestibular vessels actively regulates blood flow. Furthermore, recent data suggest that even under pathological conditions, such as acute focal cochlear microcircular disorder, formation of endogenous NO plays a key role in maintaining the cochlear blood flow (12).

**Inducible type of NOS (NOS II)**

NOS II has not been detected in the normal inner ear in general. This inducible type of NOS was first recognized in the vestibular epithelium by Takumida and Anniko (13) after intratympanic injection of lipopolysaccharide (LPS). The expression of NOS II in the inner ear has been further confirmed under pathological conditions such as following inoculation of LPS (14,15), gentamicin (16) and cisplatin (17), into the hydropic ear (18,19), in ageing (20), following immune response (21), etc. and may play an essential role in pathological damage of the inner ear (22).

Under pathological conditions, NOS II was localized in inner and outer hair cells representing nerve fibres and synaptic nerve endings, phalangeal dendrites of Deiters' cells pointing to the cuticular membrane, Hensen's cells, the cells lining scala tympani, the stria vascularis, nerve fibres, spiral ganglion cells, etc. in the cochlea. In the vestibular end organs, NOS II is induced in the cytoplasm of both type I and type II cells, dark and transitional cells, the nerve fibres and vestibular ganglion cells (13-22).

**Direct detection of NO production site**

There are two general approaches to the investigation of NO-producing sites. One possible way is to investigate the localization of NOS by using NADPH-diaphorase histochemistry or immunohistochemistry for NOS, instead of direct detection of NO. Recently, Kojima et al. (23,24) reported a useful method for real-time analysis of intracellular NO in living smooth muscle cells by using 4,5-diaminofluorescein diacetate (DAF-2DA). This compound, an ester, is cell permeable and is hydrolyzed by intracellular esterase, generating 4,5-diaminofluorescein (DAF-2). DAF-2 is a new fluorescent indicator of NO. Green-fluorescent triazolofluorescein (DAF-2 T), formed by reacting NO with DAF-2, is highly sensitive to NO (detection limit: 5 nM). The fluorescence in the cells increased in proportion to the concentration of NO. We have successfully applied this method to detect NO in the inner ear in vitro (25, 26). The results clearly indicate that NO is produced in the vestibular sensory cells, dark cells, hair cells in the organ of Corti, stria vascularis and supporting cells of the organ of Corti, etc. Furthermore, it has been stated that NO is produced mainly by a constitutive type of NOS in normal animals (25, 26). This assay system is useful for continuous surveillance of intracellular NO dynamics in in vitro systems, e.g. isolated vestibular end organ, isolated organ of Corti, isolated
vestibular and cochlear hair cells, etc. However, some problems remain when it is applied to cells located within tissues or organs, or when two types of NO-producing cells are in close proximity. In this case, it is very difficult to separate the fluorescence signals from neighbouring cells, even with a confocal laser scanning microscope. Recently, it has been stated that tissues loaded with DAF-2DA show intense fluorescence when fixed in paraformaldehyde or glutaraldehyde (27). This allows us to detect intracellular NO in fixed cells. Consequently, we assessed the procedures for detecting intracellular NO by using DAF-2DA and glutaraldehyde in the guinea pig inner ear (28). The results revealed that the combination of glutaraldehyde fixative with DAF-2DA allows us to observe intracellular NO production sites even in fixed material. In fact, we can investigate NO production sites more precisely. NO was produced in a variety of cells in the inner ear. Basically, NO-producing cells are identical to NOS-immunopositive cells. According to the results from DAF-2DA histochemistry, both vestibular and cochlear hair cells can produce NO. This has been also suggested by an DAF-2DA study using isolated vestibular and cochlear hair cells (25, 26).

**NO production under different conditions**

In order to establish the possible role of NO in pathological conditions, the production of NO under different conditions was investigated by using DAF-2DA.

In normal conditions, NO was produced in different types of cells such as sensory cells in the vestibular end organs, inner and outer hair cells in the organ of Corti, etc. After adding 1 mM L-Arg to the medium, NO from the organ of Corti and utricular maculae increased time-dependently to a maximum within 40 minutes. This increase could be suppressed by both a non-specific NOS inhibitor, L-NAME and a specific inhibitor for NOS I, 7-nitroindazole (7-NI), but was not inhibited by a specific inhibitor of NOS II, S-ethylisothiourea (EIT). These findings may indicate that NO
is produced mainly by a constitutive type of NOS in normal animals (25, 26). Application of glutamate, NMDA, AMPA, gentamicin or LPS also increased the production of NO. These increases could be suppressed by both L-NAME and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801) (22, 29).

Isolated inner and outer hair cells and vestibular sensory cells were also used to confirm the suspected production of NO in the sensory cells. After addition of L-Arg, glutamate, NMDA, AMPA, or gentamicin, NO was produced from the sensory cells, while there was no significant production in the surrounding tissue, i.e. supporting cells (22, 25, 26, 29).

In the pathological, NOS II-induced animals, the production of NO significantly and rapidly increased to reach a maximum within 20 minutes. This increase was suppressed by EIT, while less inhibited by 7-NI, indicating that an increase in NO production in the pathological condition could be due to the expression of NOS II in the inner ear (25, 26).

Possible role of NO in pathophysiology of inner ear

Evidence has been gathered that NO might well play a role in the pathophysiology of the inner ear. From continuing investigations, a working hypothesis of otoneurotoxicity has been modelled (Figure 1) (22). Under pathological conditions, NO from a cNOS and its related excitotoxicity may mediate ototoxicity in the early phase, whereas NO from NOS II may contribute to the late phase of tissue damage in the inner ear. At NMDA receptors, glutamate triggers the opening of cation-permeable channels in the sensory cells. Extracellular Ca$^{2+}$ could pass through the hair cell membrane via the voltage-dependent Ca$^{2+}$ channels, concomitant with depolarization induced by activation of AMPA/kainate receptors. The entry of Ca$^{2+}$ through these channels into cells stimulates NOS activity by binding to calmodulin, which is a co-factor for NOS. Actually, in the inner ear, application of glutamate and NMDA caused a rapid but transient increase in intracellular calcium concentration in a dose-dependent manner. This NO production is confirmed in vitro using DAF-2DA (29). After glutamate was added to the medium, the NO production of the utricular maculae and Corti’s organ increased. Again, this increase could be suppressed with L-NAME. Application of NMDA or AMPA also induced an increase in NO production, though only about half of that after stimulation with glutamate. A specific non-competitive NMDA antagonist, MK-801, blocked the response to glutamate. When the AMPA antagonist 6,7 dinitroquinoxaline-2,3-dione (DNXO) was perfused, the glutamate response was similarly inhibited, though less than by MK801 (29). The synthesis of NO by NOS mediates the effects of the NMDA, quisqualate/AMPA, and kainate receptor subtypes. This involvement of NO in the early phase has also been confirmed after stimulation by LPS (22) and gentamicin (30) as well as cochlear injury induced by transient focal anoxia (31).

In a late phase of tissue damage, however, NO from NOS II may exacerbate the tissue damage. Recently, expression of NOS II has been demonstrated in the inner ear as described above. It is a well known fact that, once induced, NOS II can produce larger amounts of NO for prolonged periods of time. Prolifer NO production by inner ear NOS II-positive cells can damage adjacent tissues. The significant increase in NO production in pathological animals may support the hypothesis that, even in the inner ear, NOS II is actually expressed following adequate stimulus, resulting in a profuse production of NO, which may also be involved in diseases of the inner ear (14-22).

A major action of NO is its activation of guanylate cyclase, which stimulates the production of cyclic GMP. Excess cyclic GMP depresses electric coupling, increases the spontaneous firing rate of neurons and stimulates further excitatory amino acid (EAA) release. Considerable attention has been paid recently to the rapid reaction of NO in aqueous solution with superoxide anion, yielding peroxynitrite. The direct toxicity of NO is modest but is greatly potentiated by reacting it with superoxide to form peroxynitrite. The increased accumulation of nitrotyrosine in the inner ear indicates generation of peroxynitrite in the pathological process (32-34). It can therefore be concluded that increased production of NO (from cNOS in early phase and from NOS II in late phase) and subsequent generation of peroxynitrite may be important factors responsible for the injury of the inner ear.

Neuropharmacology

If the activation of NO-dependent mechanisms is blocked by appropriate inhibitors or scavengers, then the inner ear is protected from the degenerative process (22). Actually, a number of drugs that modify NO-dependent mechanisms have been proposed for prevention of inner ear damage. Reactive oxygen species (ROS) scavengers and glutamate antagonists have been shown to provide protection from cochlear disruption by cisplatin, aminoglycoside, noise and ischemia/reperfusion injuries (35-37). Inner ear damage in the inflammatory process is also blocked by ROS or glutamate antagonists. Furthermore, such damage is also prevented by NOS inhibitors or scavengers of peroxynitrite (38-43). In our experiments in the guinea pig, intratympanic injection of bacterial LPS impaired caloric responses and caused severe and widespread morphological damage to vestibular end organs. These effects could be blocked with L-NAME, a competitive inhibitor of NOS, by applying superoxide dismutase, an O$_2^-$ scavenger, with dexamethasone,
and with ebsele, a scavenger of peroxynitrite. Scanning electron microscopic study revealed that the ototoxic effects of gentamicin could be blocked with L-NAME, superoxide dismutase, and with ebsele (38). Exotoxin A, produced by Pseudomonas aeruginosa, penetrates from the middle ear into the cochlea and causes sensorineural hearing loss. The hearing impairment detected by ABR was successfully blocked by applying L-NAME (39).

Clinical application of radical scavenger

As has been described above, free radicals may play an important role in inner ear disorders and free radical scavengers can be used not only for prevention but also for treatment of inner ear disorders (22, 44). Concerning Ménière’s disease, Horner and Guilhaume (45) suggested that oxidative insult probably contributes to the pathology associated with endolymphatic hydrops and that free radical scavengers might be useful in the treatment of Ménière’s disease patients. In addition, Shinomori and Kimura (46) reported that allopurinol, a xanthine oxidase inhibitor and free radical scavenger, may attenuate endolymphatic hydrops and cell damage by preventing the formation of free radicals or scavenging free radicals. These findings may lead to a new approach for the treatment of Ménière’s disease. Based on these results, we carried out a pilot study to determine whether radical scavenger could be used to treat Ménière’s disease patients who fall into the "poor prognosis" category, i.e. those who do not respond to conventional medical treatment. Radical scavengers, i.e. rebamipide (300mg/day), vitamin C (600mg/day) and/or glutathione (300mg/day) were given orally for at least 8 weeks to 25 patients with poorly controlled Ménière’s disease. After radical scavenger therapy, 21 out of 22 patients showed marked improvement of vertigo. Twelve out of 27 ears showed improvement of hearing disorders. Seventeen out of 27 ears showed improvement of tinnitus. Eighteen of 27 patients showed improvement of disability. From these results, it has been suggested that treatment using radical scavengers could potentially become an effective new therapy for Ménière’s disease (47).

Conclusion

Significant advances have been made in our understanding of the functional significance of NO in the inner ear. NO acts as an important neurotransmitter and may play an important role in the regulation of inner ear blood flow. A number of studies now suggest that NO plays an essential role in the pathophysiology of the inner ear. These include aminoglycoside and cisplatin ototoxicity, acoustic trauma, inflammation, immune response, endolymphatic hydrops, ageing, etc. Based on these results, new pharmacological strategies can be devised to prevent and/or treat inner ear disorders. Finally, the study to elucidate the functional significance of NO in the inner ear is still proceeding and will give us greater insight into forms of treatment for hearing and vestibular disorders.

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