The electrochemical and fluorescence detection of nitric oxide in the cochlea and its increase following loud sound

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Abstract

A nitric oxide (NO)-selective sensor (tip diameter 30 μm) was inserted into the perilymph of the basal turn of the guinea pig cochlea. The basal level and stimulation-induced changes of NO were measured. The mean (±S.E.M.) basal level of NO was 273 ± 42.9 nM. Following perilymphatic perfusion of the artificial perilymph containing NO synthase (NOS) substrate l-arginine (100 μM) combined with cofactor (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (100 μM), a rapid and significant increase of NO to a mean concentration of 392 ± 32.3 nM (P < 0.01, n = 10) was recorded. In contrast, a significant decrease of mean NO concentration to 180 ± 32.7 nM (P < 0.01, n = 10) was observed following the perfusion of the NOS-inhibiting agent N G-nitro-l-arginine methyl ester (100 μM). No change in the NO concentration was found following the perfusion of either artificial perilymph or N G-monomethyl-D-arginine (100 μM) solution employed as controls. Broadband noise exposure (3 h/day at 120 dBA SPL) for three consecutive days produced an increase in NO concentration to 618 ± 60.7 nM (P < 0.05, n = 10) in the perilymph. In addition, by using specific dyes for NO, 4,5-diaminofluoresceine diacetate and for the reactive oxygen species (ROS), dihydrorhodamine 1,2,3, the distribution of NO in the whole mounts of the organ of Corti and the production of ROS in vivo in the organ of Corti were investigated in both control (n = 5) and noise-exposed (n = 5) animals. The more intense NO and ROS fluorescence was observed in both the inner and outer hair cells in the noise-exposed groups. It is proposed that both the basal level and the increase in NO concentration following the addition of substrate (l-arginine) are produced by the constitutive NOS while the elevated NO and ROS following noise exposure indicate that NO may be involved in noise-induced hearing loss. © 2002 Elsevier Science B.V. All rights reserved.

Key words: Nitric oxide electrode; Polarography; Reactive oxygen species; N G-Nitro-l-arginine methyl ester; l-Arginine and (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride; Noise exposure; Guinea pig

1. Introduction

It is now widely accepted that nitric oxide (NO) plays diverse roles in physiological and pathological processes that have been elucidated over the past two decades, including neuronal signal transduction, the endothelium-dependent regulation of blood vessel tone, and the participation in both cellular apoptosis and excitotoxicity (Agnati et al., 1995; Dawson et al., 1998; Blottninger, 1999; Gosepath et al., 2000). The controlled production of a specific level of NO in certain tissues is thought to be particularly critical in these various processes (Michel et al., 1999; Fessenden et al., 1999; Reuss et al., 2000).

All three NO synthase (NOS) isoforms, i.e. neuronal isoform (nNOS), endothelial isoform (eNOS), and inducible isoform (iNOS), have recently been shown to be widely distributed in the mammalian cochlea by means of immunohistochemical, histochemical and biochemical methods (Fessenden et al., 1994; Reuss, 1998; Reuss et al., 2000; Michel et al., 1999; Wantanabe et al., 2000).
diaminofluoresceine diacetate (DAF-2DA), we previously demonstrated the distribution of NO in different cells of the cochlea (Shi et al., 2001), including the afferent nerves and their putative endings near the inner hair cells (IHCs), the efferent nerve endings near the outer hair cells (OHCs), within both the IHCs and OHCs, and in the endothelial cells of blood vessels of the spiral ligament. These findings suggest that NO may play various and important roles in the normal physiology of the peripheral auditory system. However, a number of investigations have also shown that biochemical reactions involving NO can produce toxic, especially by forming peroxynitrites (ONOO⁻), a reactive oxygen species (ROS) (Lipton, 1999; Halliwell et al., 1999). Additionally, it is thought that increased glutamatergic activity resulting in excessive NO production may be an important cytotoxic mechanism involved in sound trauma (Fessenden and Schacht, 1998) although the precise mechanisms still remain unclear. Thus, the direct measurement of NO production in the cochlea could be important both in documenting and in further elucidating the role of NO in pathophysiological processes within the peripheral auditory system.

In the current study, we detected, in vivo and in vitro, the basal level of NO as well as changes in NO concentration under various experimental conditions by using electrochemical and fluorescent dye methods.

2. Materials and methods

2.1. Subjects

Experiments were performed on 30 albino guinea pigs (both sexes, 200–250 g). All animals were obtained from the Charles River Laboratories and their outer/middle ears were confirmed to be healthy by otomicroscope. All animals had a positive Preyer reflex. The animals were divided into four groups: (1) 10 animals were used to electrochemically determine the basal level of NO and the changes in NO levels following the application of NOS stimulants and inhibitors; (2) 10 animals were used to determine the NO level following noise exposure; (3) five animals were used to measure the distribution of NO and ROS within the organ of Corti using fluorescent dyes; (4) five animals were used as controls. All procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University.

2.2. Surgery

The animals were anesthetized with an intraperitoneal injection of ketamine HCl (40 mg/kg) and xylazine (10 mg/kg). All animals were tracheotomized and breathed naturally. During the experiment the animal was lying on its back and rectal temperature was maintained at approximately 38°C by means of a thermostatically controlled heating blanket. The left bulla was opened via a lateral and ventral approach and the middle ear muscles or tendons were transected. Two small fenestrations into the cochlea were made by thinning the bony cochlear wall over the scala tympani of the basal turn. An NO sensor with a diameter of 30 μm and a length of 0.5 mm (ISO-NOP 3005, WPI Inc.) was inserted into the perilymph via one of the fenestrations, while a perfusion micropipette was inserted into the other fenestration and sealed in place with tissue cement and connected to a micropump (see Fig. 1).

2.3. Calibration of the NO electrode, measurement of NO and local perfusion of the cochlear basal turn

The production of NO in perilymph was detected by using a NO electrode at the polarization voltage of +0.8 V (plateau checked by current/voltage plot) and an electrometer (Transidyne General Corporation Model 1200). Before NO was measured, the NO probe was calibrated in a 100 ml saturated solution of cuprous chloride in a beaker in accordance with the manufacturer’s instructions. (1) A saturated solution of cuprous chloride (solution #1) was prepared by adding 150 mg CuCl₂ to 500 ml of distilled water previously deoxygenated with pure nitrogen gas for 15 min; (2) a standard S-nitroso-N-acetyl-d,l-penicillamine (SNAP) solution (solution #2) was prepared by dissolving 5 mg EDTA in 250 ml distilled water deoxygenated as described above and adjusted to pH 9.0 with 0.1 M NaOH to
which 5.6 mg SNAP was added; (3) the NO probe was calibrated in a 100 ml #1 solution stirred in a beaker by adding 20, 40, 80, 160, 320 and 640 \( \mu l \) sequentially of the SNAP stock solution #2 (Zhang et al., 1999). The current (pA) passed through the electrode was recorded with a digital chart recorder and current values were plotted against NO level. In our study, we determined the baseline NO level by placing the NO electrode into Ringer’s solution (pH 7.4; temperature 38°C) and recording the small residual (artifact) output current. This current was subtracted from the NO measurement recorded after moving the electrode into the perilymph in the cochlea. The baseline current of the electrode was checked for ‘drift’ following perilymphatic measurements by placing the electrode again into Ringer’s solution. The production of NO in perilymph was detected in the control group before and following the perilymphatic perfusion of Ringer’s solution that had a composition of (in mM): NaCl, 137; KCl, 5; CaCl\(_2\), 2; NaH\(_2\)PO\(_4\), 1; MgCl\(_2\), 1; glucose, 11; NaHCO\(_3\), 12, or Ringer’s containing (1) \( \text{N}^G \)-monomethyl-D-arginine (D-NMMA, 100 \( \mu M \)), (2) the NOS substrate L-arginine and NOS cofactor (6\( R \))-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH\(_4\), 100 \( \mu M \)), (3) the NO inhibitor \( \text{N}^G \)-nitro-L-arginine methyl ester (L-NAME, 100 \( \mu M \)). The pH of each solution was adjusted to 7.4. Perfusions were carried out at a rate of 2.5 \( \mu l/min \) for 10 min into hole A in Fig. 1 in the basal turn of the scala tympani. The perfusate was allowed to drain from the cochlea through an effluent hole placed in the basal turn of the scala tympani (hole B in Fig. 1). The effluent was absorbed within the bulla using small cotton wicks. Thus, the NO levels were measured in the perilymph in real time. The first perfusion was of Ringer’s solution alone so that later drug-related changes could be compared to a perfusion control. For the noise exposure group, the NO values were recorded after the third day of sound exposure.

### 2.4. Auditory testing

Animals were anesthetized as described above. Each ear of each guinea pig was stimulated separately with a closed sound system sealed into the ear canal. Pre-exposure thresholds were evaluated 1 day prior to the initiation of sound exposure by auditory brainstem responses (ABRs) to tone bursts at 4, 8, 12, 16, 24 and 32 kHz. Post-exposure thresholds at the same frequencies were obtained immediately after the third day of sound exposure. The ABR procedure followed the experimental design of Mitchell et al. (1996). Body temperature was monitored via a rectal probe and maintained around 38°C throughout the procedure.

### 2.5. Noise exposure

Fifteen animals were exposed 3 h/day to broad band noise at 120 dB SPL (A-weighted) for three consecutive days. Each guinea pig was placed in a sound exposure booth in front of the speaker.

### 2.6. Fluorescent assays for in vivo determination of ROS and in vitro determination of NO

#### 2.6.1. In vivo assays of ROS following noise exposure

Levels of ROS were assessed in vivo by loading cells with 20 \( \mu M \) dihydrorhodamine 1,2,3 (DHR 1,2,3) for 40 min (by the above-described method for perfusion) in the perilymph. Images of the left cochlear partition were then taken by intravital fluorescence microscopy with a 20\( \times \) objective lens (0.4 NA) (Fig. 2). Images of cells of the organ of Corti loaded with DHR 1,2,3 were collected and stored on computer hard disk by custom-written video analysis software. Quantiﬁcation of DHR 1,2,3 ﬂuorescence was achieved by measuring the ﬂuorescent intensities using three deﬁned standard areas, sizes of 8\( \times \)9 \( \mu m \) for IHCs, 20\( \times \)20 \( \mu m \) for OHCs and 11\( \times \)10 \( \mu m \) for non-cellular background (to correct for differing background intensities). All image analysis was carried out using Adobe Photoshop\textsuperscript{®}. The data were averaged within the five experimental and five control animals. Since the tissues examined varied slightly from animal to animal, we took steps to control for variability of the results: (a) care was taken to ensure consistent dye-loading times; (b) all images were illum-
nated using the same light intensity and recorded using the same settings.

2.6.2. In vitro measurement of NO following noise exposure

The right auditory bulla was dissected; the second turn of the organ of Corti was removed. It was then incubated in a petri dish in a physiological solution equilibrated with 95% O₂/5% CO₂ at 37 ± 1°C, pH 7.4, containing DAF-2DA (10 μM), dye-loaded for 30 min and then washed in a fresh physiological solution for 10 min. Observation was carried out with a fluorescence microscope and images were recorded and analyzed using Metamorph® software (Shi et al., 2001).

2.7. Statistical analysis

Mean (± S.E.M.) values of NO, ROS, and ABR were compared using a two-tailed Student’s t-test. Statistical significance was defined as P≤0.05.

3. Results

3.1. Calibration of electrode

The calibration of the NO electrode was performed by the addition of the NO donor, SNAP, into the CuCl₂ solution (the above-described method). This generated NO causing a redox current to be passed through the electrode. Fig. 3 illustrates one example calibration plot.

3.2. Typical recordings of NO production in perilymph following perfusion NOS substrate and inhibitor

Fig. 4A–D shows the typical response of the NO electrode following perfusions of Ringer’s solution, d-NMMA (100 μM), L-NAME (100 μM) and L-arginine (100 μM) combined with BH₄ (100 μM), respectively. All recordings were from one animal. Fig. 4A,B shows only a slight change of NO following perfusion with the control solutions. Fig. 4C shows the reduction of the NO output current from 300 pA to 170 pA (NO level from 320 nM to 187 nM) following the perfusion of the NOS inhibitor L-NAME (100 μM). Fig. 4D shows the elevation of the NO current from 300 pA to 410 pA (NO level from 320 nM to 450 nM) after administration of NOS substrate.

3.3. The basal level of NO and changes of NO in the cochlear perilymph following NOS substrate and NOS inhibitor

The mean value 273 ± 43 nM for the basal level con-
centrations of NO released from different cells in the cochlea was determined. A rapid and significant increase of NO was observed after perfusion of the perilymph with both L-arginine (100 μM) and BH$_4$ (100 μM), with a mean NO concentration of 392 ± 32.4 nM being recorded ($P < 0.05$, $n = 10$). A mean decrease in NO concentration to 180 ± 32.70 nM was observed following the perfusion of the NO-inhibiting agent, L-NAME (100 μM) ($P < 0.01$, $n = 10$). No change in the NO concentration was found following the perfusion of either artificial perilymph or the inactive form of the blocker, d-NMMA (100 μM) solution (as shown in Fig. 5).

3.4. ABR thresholds before and after noise exposure

ABR thresholds measured after 120 dB SPL noise exposure are shown in Fig. 6. Animals showed thresholds which were 42–50 dB higher than their initial thresholds across a broad frequency range. There were statistical differences between before and after thresholds at each measured frequency ($P < 0.01$, $n = 10$ as shown in Fig. 6).

3.5. Noise-induced changes in perilymphatic NO level

To determine whether NO production was altered following noise exposure, NO levels were measured after 3 days of noise exposure in 10 animals. We found a significant, mean increase to 618 ± 60.7 nM in NO production immediately following exposure to broad band noise at 120 dB SPL (A-weighted) for three consecutive days (as shown in Fig. 7).

3.6. NO signal in the organ of Corti before and after noise exposure

Morphological examination of the cochlea was performed in vitro by fluorescence microscopy. Fig. 8A gives a typical example of the observed NO fluorescence in both the IHCs and OHCs of the control tissue. In contrast, relatively irregular NO signals were found in the noise-exposed group. IHCs and afferent nerve endings typically have a higher fluorescence intensity than other cells of the control tissue. In the noise-exposed

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** Averaged data from 10 animals showing the basal NO level and NO released into perilymph perfused with Ringer's solution and d-NMMA (controls), t-NAM (an inhibitor for NOS) and t-arginine combined with BH$_4$ (substrate for NO). The basal release of NO was significantly attenuated by the NO inhibitor, t-NAM (fourth bar), and significantly increased by t-arginine and BH$_4$ (fifth bar) (*$P < 0.01$, $n = 10$).

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Significant ABR threshold shifts were observed in the range of frequencies 4–32 kHz following exposure to a wide-band noise at 120 dB SPL (A-weighted, 3 h/day for 3 days). Thresholds were 42–50 dB higher than their initial thresholds ($P < 0.01$, $n = 10$). The solid line indicates the mean thresholds measured prior to noise exposure. The broken line indicates thresholds that were measured immediately after noise exposure. The bars show standard deviations.

![Fig. 7](https://example.com/fig7.png)

**Fig. 7.** The level of NO following noise exposure in the control and noise-exposed animals. A significant increase in mean level of NO occurred following exposure to a wide-band noise at 120 dB SPL (A-weighted), 3 h/day for 3 days (*$P < 0.01$, $n = 10$).
tissue, hair cells (HCs) have the higher fluorescence. In addition, OHCs appear to be missing (as shown in Fig. 8B).

3.7. Activity of intracellular ROS

In control animals, only relatively weak fluorescent signals were observed in the organ of Corti (Fig. 9A), but following noise exposure, more intense fluorescence can be seen in the IHCs, OHCs, and efferent nerve fibers in the organ of Corti from animals exposed to wide-band noise (Fig. 9B). A significant increase in fluorescence intensity was detected (Fig. 10; \( P < 0.01, n = 5 \)).

4. Discussion

4.1. The basal production of NO and its chemical modulation in cochlear perilymph

The measurement of NO in biological fluids is important for investigations of the role of NO in the vascular, nervous, and immune systems. In a recent study, employing a fluorescent dye (DAF-2DA) specific for NO, we reported the distribution of NO within different cells of the guinea pig cochlea (Shi et al., 2001). In the present study, we have extended our earlier work by directly detecting changes in NO levels within the perilymph following the application of both an NOS substrate and an NOS inhibitor, as well as following wide-band noise exposure. In addition, we employed a fluorescent technique to investigate the production of NO-related ROS within the cochlea.

A basal level of NO in the cochlear perilymph was found under resting conditions. In comparison to other
of 30–270 nM reported in synaptosomes (Brown, 1995). Although it is possible that the basal level of NO might have been influenced by the experimental surgery, a certain amount of NO probably exists in the cochlea under normal physiological conditions. Since NO readily permeates tissue and can diffuse for some hundreds of microns from the source where it was generated (Pryor et al., 1997), this concentration of NO in the perilymph may be related to several factors, including: (1) the total number of NO-producing cells in the cochlea; (2) its rate of production; (3) its high diffusion constant; and (4) its half-life of chemical reactivity. The localized, quantitative findings in this study further support the hypothesis that NO is involved in important physiological functions in the cochlea (Fessenden et al., 1999; Reuss et al., 2000) probably by the well known NO/cyclic GMP/cGK-1 (protein kinase G) pathway (Dawson et al., 1998; Fessenden et al., 1999; Michel et al., 1999).

To examine the variability of the biochemical production of NO production, we first perfused the perilymph with the NOS substrate, L-arginine along with the cofactor BH4. This resulted in a rapid and significant rise of NO. In contrast, we observed a decrease of NO production when the perilymph was perfused with the non-specific NOS inhibitor L-NAME. In some pre-

![Fig. 10. A histogram comparing the intensity of ROS fluorescence in the control and noise-exposed animals. ROS activity was significantly increased in the noise-exposed group (*P < 0.05, n = 5).](image)

![Fig. 11. A cartoon showing NO production and a proposed mechanism of cochlear excitotoxicity. In the normal condition (on the left), Glu (the IHC putative neurotransmitter) is released upon acoustic stimulation and activates Glu receptors (NMDA) present on IHC afferent dendrites. Calcium enters the dendrite and binds with calmodulin to stimulate intracellular NO production. NO, a freely diffusible gas messenger molecule, then binds to and activates intracellular soluble guanylate cyclase (sGC) leading to the production of cGMP which is known to activate protein kinase G (PKG). PKG can open ion channels and affect cellular function by activating or inhibiting specific downstream targets. Overstimulation (on the right) can cause an increase in calcium influx into both HCs and afferent nerve dendrites resulting in an excess production of NO. NO can diffuse back to the IHC where it could join with locally produced NO to stimulate the release of additional Glu as a positive feedback. The excess NO can also react with superoxide radicals (O2•−) to generate other ROS, such as peroxynitrite (ONOO−). The resulting oxidative stress damages DNA and mitochondria leading to disruption of energy metabolism and calcium homeostasis causing cell necrosis and/or apoptosis (Michel et al., 1999).](image)
vious studies of non-cochlear tissues, the finding of increased NO following local application of l-arginine has been controversial because the basal intracellular level of l-arginine appears more than adequate to saturate the enzyme under physiologic conditions. Thus, it has been argued that additional l-arginine should not increase NO production (Pollock et al., 1991). However, other researchers have observed in vivo results similar to our findings, suggesting that increasing l-arginine concentration can, in fact, cause an increase in NO production (e.g. Tsikas et al., 2000). The fact that NO production within the cochlea can be enhanced or suppressed by the addition of NO substrate and NOS inhibitors suggests that NO production within the cochlea might be modulated for other experimental and/or therapeutic purposes.

4.2. Increased NO production in the cochlear perilymph and its possible role in ototoxicity following noise exposure

Following noise trauma, which produced a threshold shift of approximately 50 dB, we directly detected, with the NO-sensitive electrode, a markedly higher concentration of NO in the perilymph. To further examine the distribution of NO in the cochlea before and after noise trauma, we employed a highly sensitive, specific fluorescent indicator (DAF-2DA) that permits the direct detection of NO in tissue (Kojima et al., 1998). In contrast to the relatively uniform fluorescent intensity detected within HC s in the organ of Corti under normal conditions, significantly stronger but irregular fluorescent signals were observed in IHCs, OHCs, and afferent nerve endings, following wide-band noise exposure. Under the assumption that the observed changes in fluorescence are the result of NO production within the HC s and nerve fiber, increased fluorescence could occur through two different mechanisms: (1) an upregulation of constitutive NOS (nNOS) and/or (2) the expression of iNOS, as has been demonstrated in animals with overstimulation of the cochlea. In our current study, using a specific dye, DHR 1,2,3, to detect ROS, we compared its activity in both control and noise-exposed animals. Enhanced ROS activity in the HCs (Fig. 9B) was observed following wide-band noise exposure. This finding supports the hypothesis that excess ROS plays a role in cell injury and death accompanying hearing loss possibly by a complex process, including damaged DNA and mitochondria, the disruption of energy metabolism and calcium homeostasis and activation of cell death mechanisms (Michel et al., 1999).
In addition, the basal release of NO is known to play an important role in the regulation of vascular homoeostasis (Brechtlefbauer et al., 1994). However, excessive amounts of NO can also result in oxidative injury to vascular cells, including smooth muscle cells and endothelial cells by the inhibition of mitochondrial respiration (Dawson et al., 1992; Brown, 1995). Ototoxic effects on the stria vascularis (lower endocochlear potential) following exogenously applied NO have been reported (Kong et al., 1996). Therefore, overproduction of NO may also result in dysfunctional microcirculation in the cochlea leading to hearing loss.

Our study provides new evidence that NO production may contribute to both physiological and pathological processes in the cochlea. However, further investigation will be required to precisely define the role of NO in the auditory system and to better elucidate the precise pathophysiological role(s) of NO in noise-induced hearing loss.

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