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Nitric oxide and mitochondrial status in noise-induced hearing loss

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Abstract
This study investigated the distribution of nitric oxide (NO) within isolated outer hair cells (OHCs) from the cochlea, its relationship to mitochondria and its modulation of mitochondrial function. Using two fluorescent dyes—4,5-diaminofluorescein diacetate (DAF-2DA), which detects NO, and tetramethyl rhodamine methyl ester ($TMRM^+$), a mitochondrial membrane potential dye—it was found that a relatively greater amount of the DAF fluorescence in OHCs co-localized with mitochondria in comparison to DAF fluorescence in the cytosole. This study also observed reduced mitochondrial membrane potential of OHCs and increased DAF fluorescence following exposure of the cells to noise (120 dB SPL for 4 h) and to an exogenous NO donor, NOC-7 (>350 nm). Antibody label for nitrotyrosine was also increased, indicating NO-related formation of peroxynitrite in both mitochrondria and the cytosol. The results suggest that NO may play an important physiological role in regulating OHC energy status and act as a potential agent in OHC pathology.

Keywords: *Outer hair cells, mitochondria, nitric oxide, noise exposure.*

Abbreviations: *OHC, Outer hair cell; NO, Nitric oxide; DAF-2DA, 4,5-diaminofluorescein diacetate; $TMRM^+$, Tetramethyl rhodamine methyl ester; NOS, Nitric oxide synthase; ROS, Reactive oxygen species; NT, Nitrotyrosine; $ONOO^-$, Peroxynitrite; AIF, Apoptotic induced factor.*

Introduction

The change of mitochondrial-energy metabolism is particularly compelling in hearing loss caused by loud sound [1–4]. Mitochondria are not only important for providing cellular energy needs, but are also major producers of free radical species as well as being a major target for oxidative damage [5]. Reactive oxygen and nitrogen species have been shown to be strongly involved in damage to cochlear cells by ototoxic drugs and loud sound [6–14]. Loud sound can induce hair cell apoptosis via the caspase pathway that is initiated by mitochondrial release of cytochrome *c* [15,16] and by a non-caspase pathway by mitochondrial release of apoptotic induced factor [10,17]. The release of mitochondrial factors is a late signal of cell damage, after which cells are irreversibly committed to cell death. However, less is known about earlier events of cochlear cell damage by direct stress to mitochondria and this lack of knowledge is an obstacle to understanding loud sound damage to OHCs.

Cells produce nitric oxide (NO) from at least three different isoforms of nitric oxide synthase (NOS):

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neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). NO plays an important role in various physiological processes, including neurotransmission, immune response and vasodilatation [18]. NO can freely permeate membranes and diffuse in and out of a cell (and thus mitochondria) over its half-life span of seconds, acting as an integrator/modulator signal of complex physiological processes [19]. NO may also be directly produced from a hypothesized mitochondrial NOS (mtNOS), a possible α-isoform of nNOS, which may be localized at inner mitochondrial membranes [20,21]. Mitochondrial NO in high concentrations, whether from mtNOS or by diffusion from the cytosole, can bind and inhibit components of the respiratory chain, including the oxygen binding site of cytochrome oxidase [22], and even cause irreversible inhibition of mitochondrial respiration by damage to a variety of mitochondrial components via oxidizing reactions [19,21,23–26].

NO is increased in cochlear cells following exposure to a loud sound [27–29]. Both nNOS and eNOS immunolocalize to the electron dense area of mammalian OHC cytoplasm, where mitochondria are concentrated [28,30]. It is not known whether reactive nitrogen species such as peroxynitrite are implicated in hair cell mitochondrial damage, as might be evidenced by a suppression of the mitochondrial membrane potential. In this study, using the combination of the fluorescent intracellular NO indicator DAF-2DA and a selective marker for functional mitochondria, $TMRM^+$, we found by confocal fluorescence microscopy a high level of DAF-2DA signal within the mitochondria of sensory OHCs. Exogenously applied NO donor (NOC-7) generating the relatively low concentration of 350 nm that could depolarize mitochondria membrane potential. Loud sound exposure increased DAF-2DA fluorescence and the amount of mitochondrial-localized nitrotyrosine, a protein reaction product of peroxynitrite, in OHCs. These results support the hypothesis that loud sound exposure produces nitrogen free radicals that damage mitochondria in OHCs.

Methods

Animal preparation and noise exposure

Experiments were performed on 80 albino guinea pigs (both sexes, 200 ~ 250 g). All animals were obtained from the Charles River Laboratories and had a positive Preyer reflex. Animals in the noise exposure group were placed in wire mesh cages and exposed to broadband noise at 120 dBA SPL for 3 h in a sound exposure booth. In this study, a total of 13 albino guinea pigs received noise exposure. For each noise exposure experiment, a single animal was placed in the sound exposure booth. During noise exposure the animals had free access to food and water. This noise exposure is routinely used in our laboratory [9]. All procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University.

Auditory testing

Auditory brain-stem responses (ABR) audiometry to pure tones was used to evaluate cochlear function, following our standard experimental design [31].

For the ABR test, each animal was anaesthetized with xylazine (10 mg/kg, im) and ketamine (40 mg/kg, im) and placed on a heating pad in a sound-isolated chamber. The external ear canal and tympanic membrane was inspected using an operating microscope to ensure the ear canal was free of wax, there was no canal deformity, no inflammation of the tympanic membrane and no effusion of the middle ear. Then needle electrodes were placed subcutaneously near the test ear, at the vertex, and at the contralateral ear. Each ear of each animal was stimulated separately with a closed tube sound delivery system sealed into the ear canal. The ABR to 1 ms rise-time tone burst stimuli at 4, 8, 12, 16, 20 and 32 kHz were recorded and thresholds obtained for each ear. Threshold is defined as an evoked response of ~0.2 μV from the electrodes. This method was used to assess the pre-exposure thresholds and the ABR thresholds immediately after noise exposure (i.e. the Temporary Threshold Shift).

Calibration of the nitric oxide sensing electrode

To calibrate NO production in the bath chamber where isolated OHCs were studied, we used an NO electrode (ISO-NO, World Precision Instruments, Sarasota, FL, USA). The electrode itself was calibrated according to the manufacturer's instructions as detailed in our previous report [32] and the calibration curve used in turn to calibrate the concentration of NO in the cell recording exposure chamber is shown in Figure 1. Briefly, first a saturated solution of cuprous chloride (solution #1) was prepared by adding 150 mg $CuCl_2$ to 500 ml of distilled water previously deoxygenated with pure nitrogen gas for 15 min. Second, a standard *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP) solution (solution #2) was prepared by dissolving 5 mg EDTA in 250 ml distilled deoxygenated water and adjusted to pH 9.0 with 0.1 m NaOH to which 5.6 mg SNAP was added. Third, the NO electrode was dipped into 100 ml of solution #1 stirred in a beaker with increasing levels of NO resulting from adding 20, 40, 80, 160, 320 and 640 μl sequentially of the SNAP stock solution #2. 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 also determined the baseline zero NO level in physiological solution

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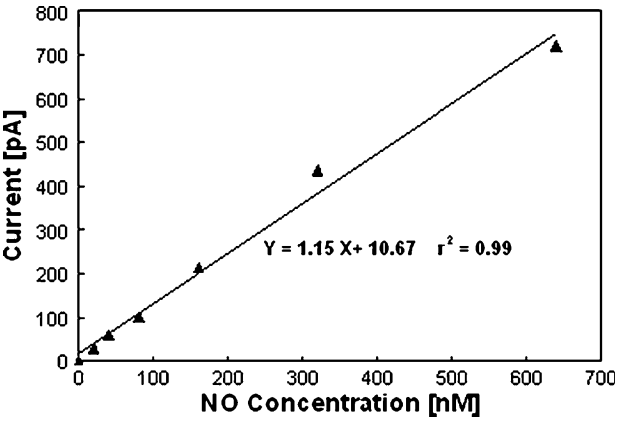

Figure 1. A typical calibration profile of an NO electrode when current (pA) is plotted against the concentration of NO (nm). The increased output current was in response to the NO-donating compound (SNAP) and there was a linear relationship between the amount of NO generated by SNAP and the output current of the electrode.

(composed of (in mm): NaCl 125, KCl 3.5, glucose 5, HEPES 10, $CaCl_2$ 1.3 and $MgCl_2$ 1.5, NaH_2PO_4 0.51). The osmolarity of the solution was adjusted to 310 mOsm with NaCl and the pH was adjusted to 7.4 with NaOH, T: 37°C.

In our experiments that determined the effect of NO on isolated OHC mitochondrial potential, we added a bolus of NOC-7 to the bath. To estimate the real-time NO concentration produced by the NO donor in the physiological solution, the decomposition of the NOC-7 was monitored with the calibrated NO probe. NOC-7 was added to 150 µl of physiological solution in a petri dish to obtain a final concentration of 0.3 µm, 1 µm and 3 µm. The NO release curves were obtained, as shown in Figure 2. The shape of these curves likely arises from both the evolution of NO from the donor and the loss of NO from the bath via the surface of the solution being open to the air in the dish. To avoid disturbing the OHCs under observation the solution was not stirred.

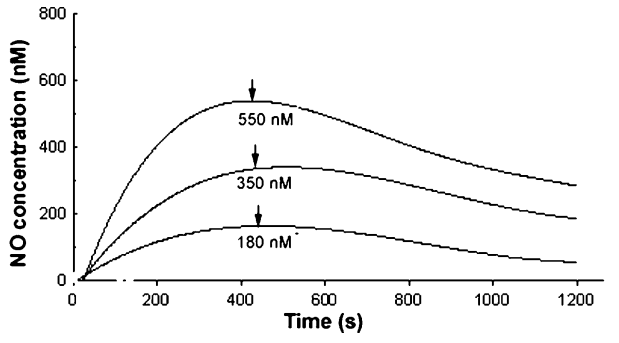

Figure 2. Concentration-time profiles of NO decomposition/depletion kinetics following the addition of 0.3, 1 and 3 µm of NOC-7 into physiological solution at pH 7.4 and 37°C. Measurement of NO generated by NOC-7. NOC-7 at 0.3 µm could generate a peak concentration of ~180 nm NO while higher concentrations of NOC-7 at 1 and 3 µm generated peak concentrations of ~350 and 550 nm NO.

Therefore, the peak NO concentration estimated the maximum level of NO that OHCs would experience.

Detection of mitochondrial NO in situ

Isolated OHCs from three guinea pigs were used to identify the mitochondria co-localizing with DAF-2DA fluorescence. Briefly, each animal was decapitated under deep anaesthesia and the bulla and the otic capsule were opened. The apical and third turns of the cochlea were immediately removed and transferred into a drop of physiological solution (osmolarity adjusted to 300 mOsm; pH was buffered to 7.4) with collagenase Type IV (1 mg/ml physiological solution) in a petri dish for 10 min. After enzyme treatment, the tissues were washed three times in fresh physiological solution. The OHCs were then isolated and transferred into 150 µl of fresh physiological solution on a cover slip chamber. The NO fluorescence indicator 4,5-diaminofluorescein diacetate (DAF-2DA, 5 µm, Calbiochem Cat 251505, LA Jolla, CA, USA) and the mitochondrial potential probe tetramethylrhodamine methylster ($TMRM^+$, 50 nm, Molecular Probes, Carlsbad, CA, USA) were used to detect the colocalization of NO production and the location of mitochondria [33,34]. A short wavelength-pass excitation filter of below 488 nm and a long wavelength-pass emission pass filter above 520 nm were used for DAF-2 DA. A short wavelength-pass excitation filter below 568 nm and a long wavelength emission filter above 615 nm were used for $TMRM^+$.

Measurement of mitochondrial potential in isolated outer hair cells with NOC-7 treatment

Isolated OHCs with mitochondria labelled by $TMRM^+$ for 30 min at 37°C were divided into two control groups and three experimental groups. Each group was from one guinea pig (of 10) and was composed of 10 individual isolated OHCs treated and imaged individually. Isolated OHCs in one of the control groups were not treated with NOC-7. The other control group was treated with the inactive NOC-7 solution created by previously adding NOC-7 (3 µm) to the physiological solution 24 h prior to use. Over the course of 24 h, the NO released dissipates to the atmosphere. The experimental groups were: (1) isolated OHCs treated with 0.3 µm NOC-7; (2) isolated OHCs treated with 1 µm NOC-7; and (3) isolated OHCs treated with 3 µm NOC-7. In order to determine specificity and uniformity of loading of DAF-2DA, which served as a NO indicator, isolated OHCs were pre-treated with NOS inhibitors: N^G-nitro-L-arginine methyl ester HCl (L-NAME, 300 µm) for 30 min before loading DAF-2DA and $TMRM^+$ dyes. To test DAF-2DA loading uniformity, we added NOC-7 at 1 µm into

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the bath for 10 min and then images were taken under a confocal fluorescence microscope.

Digital images from the different groups were taken at 0, 180, 360, 540, 720 and 900 s following exposure to the various control and experimental solutions. Photoshop® image analysis software was used to measure OHC fluorescence intensity. Mitochondria have a high level of fluorescent signal because $TMRM^+$ accumulates up to 10,000-fold in mitochondria [35]. The fluorescent intensity measurements were carried out on the frame histograms, avoiding manipulation of the images. In a typical histogram [36], it is easy to recognize (1) a very sharp peak with minimal intensity (between: $0 \sim i_1$ where the i_1 intensity is a gray level of $15 \sim 25$), corresponding to the frame background; and (2) a broader peak (partially superimposed to the background) that corresponds to the cell cytoplasm fluorescence distribution/fluorescence intensity between i_1 and i_2, where i_2 intensity is a gray level of $50 \sim 60$. Pixel values above the i_2 level correspond to the mitochondria. The percentage change of mitochondrial fluorescence under the different concentrations of NOC-7 treatment was quantitatively converted into fluorescence difference (ΔF) with respect to background level. $\Delta F\ (\%) = (MF_{it} - \text{Background})/(MF_{i0} - \text{Background}) \times 100$ (MF_{i0}: mitochondrial fluorescence before application of NO donor, NOC-7; MF_{it}: mitochondrial fluorescence over different time points after NOC-7 treatment). The percentage change of mitochondrial fluorescence signal ($\Delta F\%$) in mitochondria is a qualitative measurement of mitochondria membrane potential.

Detection of NO production and mitochondrial membrane potential in OHC of organ of Corti whole mounts following noise exposure

Cochlea were dissected from five control and five noise-exposed animals. For each experiment, a segment of organ of Corti from the second turn of the cochleae of one control animal and one noise-exposed animal were compared. The organ of Corti from the noise-exposed animal was removed immediately following the noise exposure. The tissues were incubated in the physiological solution at 37°C, pH 7.4 containing DAF-2 DA (10 μm) and $TMRM^+$ (100 nm, Molecular Probes, Inc). The dyes were loaded for 30 min. The tissues were then observed under confocal microscopy. The same settings for gain and illumination power were used for the control and loud sound stimulated organ of Corti segments. The mitochondria in the apical domain of hair cells are potentially crucial for providing energy to stereocilia for hair bundle calcium regulation during sound stimulation and for membrane protein turnover, as well as for lipid segregation [37–39]. We reasoned that mitochondria at the apical region could be more vulnerable to sound damage. Therefore, we used confocal images to show the change of mitochondrial membrane potential and DAF-2DA fluorescence at the level next to the sub-cuticular plate. Confocal optical sections of the organ of Corti at this plane give relatively high-quality images, since optical dispersion is minimal. We used one confocal optical section (5 μm into the cell from the level of the cuticular plate) for analysis. Photoshop® was used to quantify the level of DAF-2DA fluorescence (green channel) and mitochondrial membrane potential ($TMRM^+$ fluorescence) in the red channel was evaluated. For each recorded image (which are from each second turn of the organ of Corti segment obtained from one cochlea), about 30 outer hair cells were in focus at the sub-cuticular plate region. Using the Photoshop® lasso tool, either the mitochondria areas or the mitochondria-free cytosol areas were selected in 113 OHCs (control tissues from five animals) and 117 OHCs (noise-stimulated tissues from five animals). All three rows of OHCs were equally represented. The mean pixel intensities of the composite intensity histograms from the organ of Corti were then statistically compared for control and noise exposure using a non-paired two-tailed Student's *t*-test.

Detection of NO production and reactive oxygen species activity in OHC mitochondria

To determine the relationship between mitochondrial NO and reactive oxygen species (ROS) production, cochleae from three control and three noise-exposed animals were dissected and segments of organ of Corti from the second cochlear turn were removed and then incubated in a physiological solution containing DAF-2DA (10 μm) and MitoTracker Red CM-H_2XRos (100 nm, Molecular Probes, Inc). MitoTracker Red CM-H_2XRos is a reduced, non-fluorescent version of MitoTracker Red (M-7512) that fluoresces upon oxidation [40,41]. The dyes were loaded for 30 min. The tissues were then observed under confocal microscopy and fluorescence intensity was measured as described for NO production and TMRM. For MitoTracker Red CM-H_2XRos, an excitation filter passed short wavelengths below 568 and an emssion filter passed long wavelengths above 615 nm.

Nitrotyrosine immunocytochemical staining and measurement

Nitrotyrosine (NT) has been used extensively as a biomarker of NO-related peroxinitrite ($ONOO^-$) reaction with protein tyrosine residues [42]. To determine the level of NO-related reactive nitrogen species in the hair cell mitochondria, we immunochemically detected the changes of immunoreactivity of NT and its co-location with MitoTracker Red CMXRos. This dye is well retained after aldehyde

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fixation [43]. The isolated organ of Corti from six control and five noise-stimulated animals were incubated with MitoTracker Red CM-H_2 XRos 568 (100 nm) for 30 min and then fixed with 4% paraformaldehyde for 4 h. The fixed cochlear tissues were first washed in 0.02 PBS (pH 7.4), permeabilized in 0.5% Triton x-100 (Sigma) for 1 h and immune-blocked in 10% goat serum in 1% bovine albumin in 0.02 m PBS for 1 h. The specimens were incubated overnight in anti-nitrotyrosine (mouse monoclonal antibody, 39B6, Alexis biochemicals) diluted 1:500 with 1% BSA-PBS. The specimens were washed in 1% BSA-PBS for 30 min and incubated in Alexa Fluor 488 anti-mouse IgG for NT (diluted 1:100 with 1% BSA-PBS) and Alexa Fluor 568 phalloidin was added for labelling the structure of the hair cells (diluted 1:50 with 1% BSA-PBS, Molecular Probes, Inc.) for 1 h. After washing in 0.02 PBS for 30 min, the tissues were mounted and observed with confocal microscopy. Tissues incubated with 1% BSA-PBS to replace the primary antibody were the negative control. Immunofluorescence intensity for nitrotyrosine was measured with Adobe Photoshop®. Briefly, for each recorded image, the areas of sub-cuticular plate of OHCs (including three rows and a total of 30 cells) were selected with the lasso tool and then measured with the Photoshop® histogram function, obtaining a gray scale value. A background intensity was determined in a small window located away from the fluorescence of the hair cells and was subtracted from the fluorescence intensity value within the hair cells. The mean value from the individual images was summed from the three animals of each group.

Statistical analysis

Mean ($\pm$ SD) values of DAF-2DA, $TMRM^+$ NO, ROS and NT were compared using a two-tailed Student's *t*-test. Statistical significance was defined as $p \leq 0.05$.

Results

DAF-2DA fluorescence was co-localized with mitochondria in vitro

The isoforms of NOS known to exist in OHCs likely contribute to basal and stimulated levels of NO in the cells. The production of NO and its distribution in sub-cellular compartments can be investigated with DAF-2DA, a fluorescent probe for NO. Co-localization of the DAF-2DA fluorescence signal within the mitochondria was demonstrated in an isolated OHC by incubating the cells in physiological solution containing the mitochondrial membrane potential dye $TMRM^+$ (Figure 3A), together with DAF-2DA (Figure 3B). A diffuse green fluorescent signal was seen in the entire cell, but a relatively high green fluorescence signal was co-localized in mitochondria with $TMRM^+$ labelling. Image intensity segmentation analysis of the mitochondrial compartment confirms a visually obvious significantly higher fluorescence than the cytosol. We found a significantly decreased DAF-2DA fluorescence signal by treating isolated OHCs with L-NAME at 300 μm for 30 min before dye loading (see Figure 3D and E). This confirms that the DAF-2DA signal was dependent on nitric oxide synthase. To determine whether the greater DAF-2DA fluorescence in the mitochondria was due to a higher concentration of DAF-2DA trapped in the mitochondrial compartments, we treated pieces of isolated organ of Corti where OHCs were easily seen and measured OHCs with NOC-7 at 1 μm. We found a uniform DAF-2DA fluorescence throughout the cells, indicating that DAF-2DA was most likely equally distributed in the cell cytoplasm and organelle (data not shown).

Mitochondria were depolarized by exogenously applied NO in vitro

The mitochondria of different tissues and cells have different sensitivities to the effect of NO on mitochondrial activity [44,45]. Are cochlear OHCs sensitive to NO. We have found the NO concentration in the cochlear perilymph is $\sim$273 nm under unstimulated conditions and that noise exposure increases NO concentration to $\sim$393 nm [32]. To detect the sensitivity of OHC mitochondria to NO, we measured the time-dependent change of mitochondrial membrane potential following different bath concentrations of the NO donor, NOC-7. Without treatment (control A in Figure 4), we observed a slow fluorescence decrease that could be due to the dye photo bleaching. The inactive form of NOC-7 (control B) and 300 nm NOC-7 did not result in a significantly greater rate of $TMRM^+$ fluorescence decline over the 900 s observation time. However, with 1 μm NOC-7 application, $TMRM^+$ fluorescence significantly declined, approximately at 180 s of NOC-7 application. With 3 μm NOC-7 treatment, the rate of decline in $TMRM^+$ fluorescence was greater and began earlier. The mean percentage reduction of mitochondrial membrane $TMRM^+$ fluorescence was nearly 70% (NOC-7 at 1 μm) and 80% (NOC-7 at 3 μm) after 900 s of treatment (Figure 4).

Noise trauma caused hearing loss

ABR threshold shift was significant in animals exposed to noise at all test frequencies. Figure 5 shows the initial control levels (open circle symbols) and three post-sound exposure threshold curves (closed symbols). Temporary Threshold Shift (TTS) is the amount of sensitivity shift (more sound is required to evoke a response) immediately following the sound

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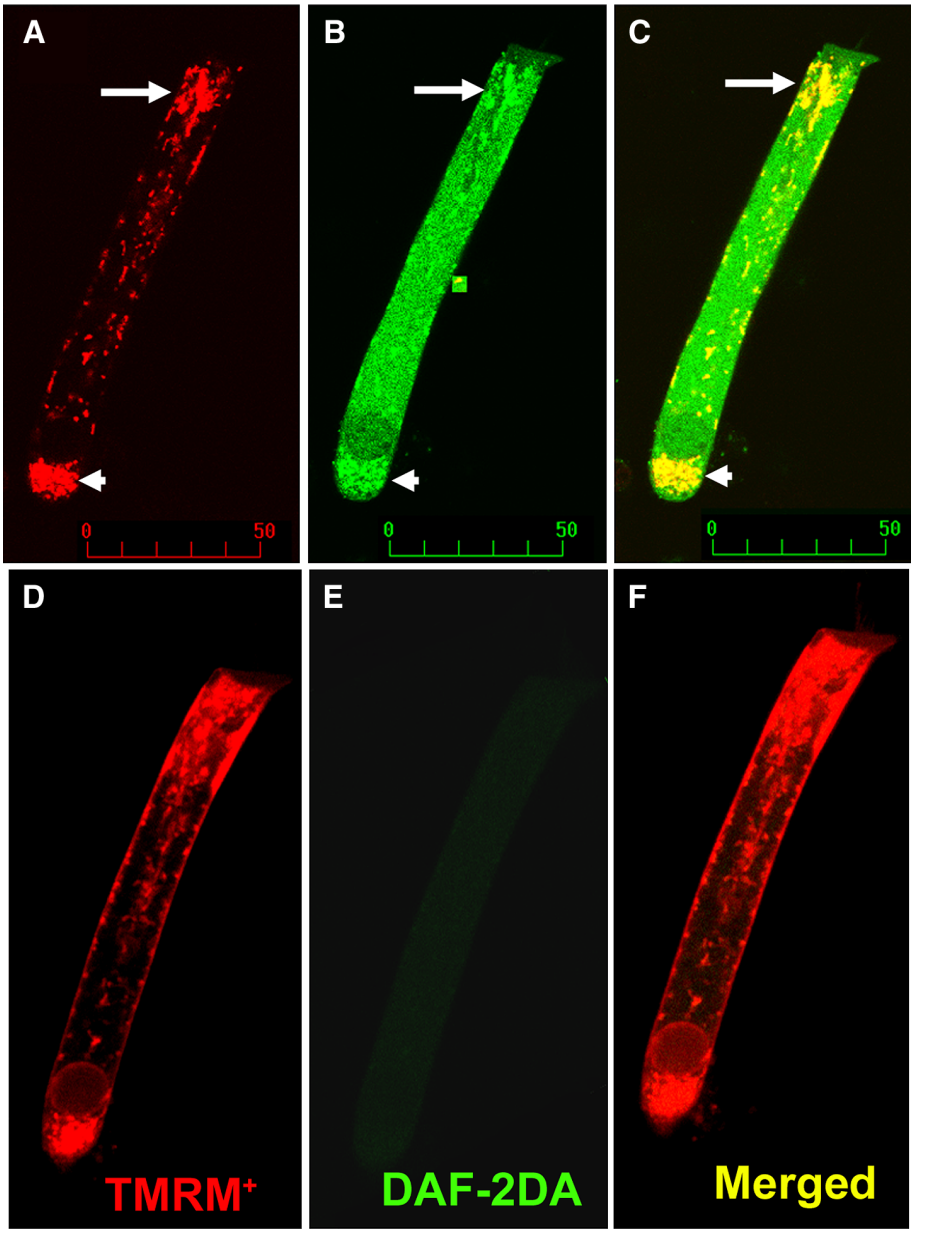

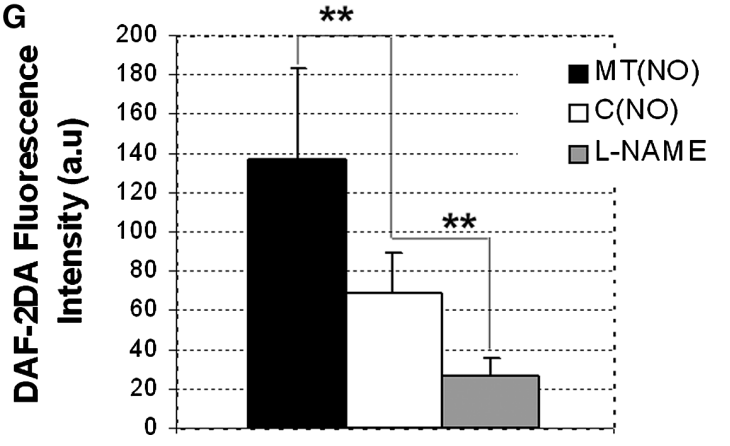

Figure 3 (*Continued*)

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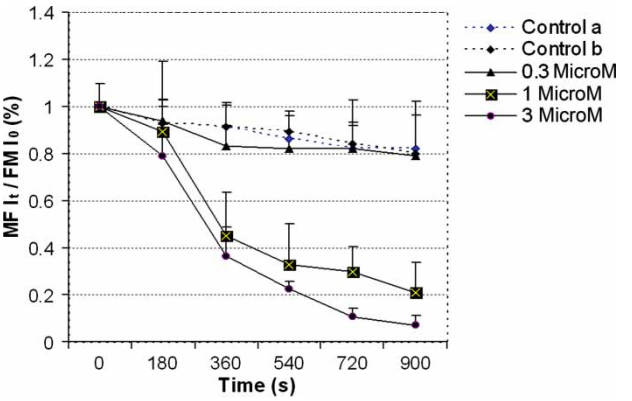

Figure 4. The normalized mean (± SD) $TMRM^+$ fluorescence changed over 900 s following application of different concentrations of NOC-7 from 10 cells in each group. Within the time course of this experiment, there was a slow decline of mitochondrial fluorescent signal when the cells were incubated in physiological solution (control a) or were exposed to 24-h-NO-depleted NOC-7 (control b). Incubation with 0.3 μm NOC-7 (peak NO of 180 nm) did not influence the decline. However, when 1 μm (peak NO of 350 nm) and 3 μm NOC-7 (peak NO of 550 nm) solutions were added to the bath, the higher concentrations of NO caused a decline of $TMRM^+$ fluorescence in the hair cells. The normalized mean fluorescent signal at 900 s was significantly different from control b. ($p_1\mu m < 0.05$; $p_3\mu m < 0.01$, $n = 10$).

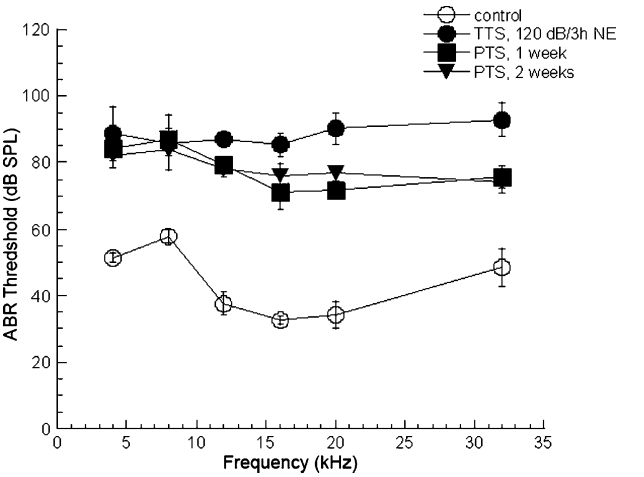

Figure 5. Noise-induced hearing loss, estimated using auditory brain-stem response thresholds before and immediately after noise (broadband noise at 120 dB SPL, 3 h) at 4, 8, 16, 20, 32 kHz. Asterisks indicate a statistically reliable difference ($p < 0.001$) between before and after noise exposure.

stimulation. However, this exposure level does result in permanent threshold shift and loss of OHCs (data not shown). Noise trauma caused increased endogenous NO production and induced mitochondria depolarization in OHCs.

Loud sound can increase cochlear NO production [32]. It is not known, however, whether sound-exposed OHCs show increases in NO, decreased mitochondrial membrane potential. We determined the change of mitochondrial membrane potential for those mitochondria concentrated in the apical area of OHCs from whole mounts of the organ of Corti. As shown in Figure 3, the population density of mitochondria in the apical area is high. The thickness of the organ of Corti makes it more difficult to obtain clear confocal microscopy images of the basal area of OHCs for analysis of mitochondria in the basal area. We therefore studied mitochondria only in the apical area. Figure 6 shows images of typical whole mounts of the organ of Corti from the cochlear second turn. In the control condition (without noise exposure), we found relatively weak DAF-2DA fluorescent signals in the mitochodnria in the apical area where the high mitochondrial population gives high $TMRM^+$ signals (Figure 6A and B). In contrast, when animals were exposed to the noise protocol, we observed a lower level of $TMRM^+$ fluorescence and a higher level DAF-2DA fluorescence as indicated by arrows (Figure 6D and E).

OHC mitochondrial DAF-2DA fluorescence increase following loud sound was co-localized with MitoTracker Red CM-H_2 XRos

NO can react with superoxide to form reactive free radicals in the mitochondrial matrix [46]. To determine whether the increased DAF-2DA fluorescence was correlated with a greater amount of ROS production, we used MitoTracker Red CM-H_2 XRos. We found relatively weak MitoTracker fluorescence along with low DAF-2DA fluorescence in the apical mitochondrial area of control OHCs from the organ of Corti of the second cochlear turn (Figure 7A and B). In contrast, following noise exposure, increased fluorescence signals of both DAF-2DA and MitoTracker Red CM-H_2 XRos were observed (Figure 7D and E). The difference in fluorescence intensity was significant between control and noise-stimulated OHCs.

Figure 3. Confocal microscopy images of an isolated OHC showing DAF-2DA fluorescence and the mitochondria labelled by $TMRM^+$. (A) A single optical section shows an isolated OHC labelled with $TMRM^+$. The arrow and arrowhead point out that mitochondria is concentrated in the apical and basal poles of a hair cell. There is also a more sparse distribution along the length of the cell. (B) DAF-2DA fluorescence, indicating the NO in the mitochondria and cytosol (arrow and arrowhead point to DAF-2DA fluorescence accumulated in the apical and basal poles of the hair cell respectively). (C) $TMRM^+$ labelled mitochondria co-localized with DAF-2DA fluorescence (yellow area). (D) An isolated OHC labelled with $TMRM^+$ after treatment with L-NAME for 30 min. (E) Significantly decreased DAF-2DA fluorescence in this isolated OHC. (F) $TMRM^+$ labelled mitochondria co-localized with DAF-2DA fluorescence. (G) The mean (± SD) relative fluorescence intensity of DAF-2DA in the mitochondria is significantly greater than the cytosol ($n = 7$ OHCs; $p < 0.01$) and DAF-2DA fluorescence signals are significantly decreased in the entire isolated OHCs when treated with non-selective NOS inhibitor L-NAME ($n = 6$ OHCs; $p < 0.01$).

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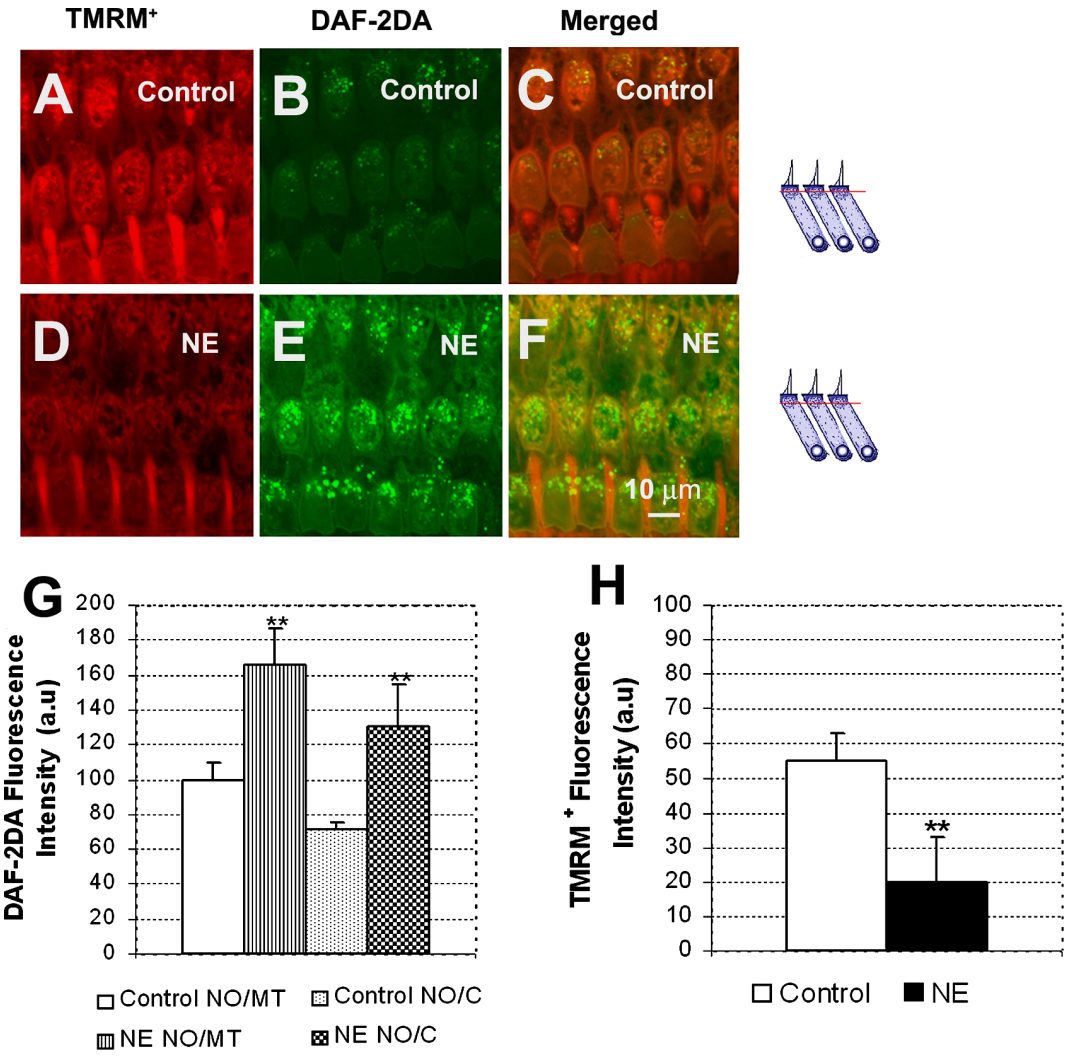

Figure 6. DAF-2DA fluorescence of whole mount in OHCs organ of Corti. (A) $TMRM^+$ fluorescence signalling in the mitochondria of the apical pole of OHCs from a control tissue; (B) DAF-2DA fluorescence signals located at the mitochondria and cytosol of the apical pole of OHCs from the same double-labelled control tissue; (D) Decreased $TMRM^+$ fluorescence in the mitochondria of the OHCs from the organ of Corti noise-stimulated tissue; (E) The increased DAF-2DA fluorescence signals in mitochondria and cytosol of OHCs from the organ of Corti from noise-stimulated tissue; (C) and (F) are merged images from the first two rows. The drawing to the right of (C) and (F) depicts a row of three OHCs with a horizontal line indicating the location of the confocal optical section at the apical level where mitochondria are concentrated. (G) A bar graph showing the mean levels of DAF-2DA in the mitochondria (MT) and cytosol (C) under control and noise-exposed hair cells. Data are means $\pm$SD ($n_{control}=113$ OHCs pooled from five specimens of five control animals, $n_{NE}=117$ OHCs pooled from five specimens of five control animals), for NO in MT of noise exposed compared to control $p<0.01$. For NO in cytosol of noise-exposed cells compared to control p was <0.01 (H) A bar graph showing the mean levels of $TMRM^+$ fluorescence under control and noise-stimulated conditions. Data are means $\pm$SD ($n_{control}=113$ OHCs; $n_{NE}=117$ OHCs, $p<0.01$).

Nitrotyrosine was increased in the apical domain of OHCs following noise exposure

Nitrotyrosine (NT) is a nitration product of proteins exposed to the NO derivative peroxynitrite ($ONOO^-$). NT is a marker of nitrositive stress and has been observed in various pathological conditions or diseases [47,48]. In this experiment, we tested whether noise exposure would result in nitration of tyrosine amino acid residues of OHC mitochondrial proteins. We used immunocytochemistry to find the areas of NT under control and noise exposure conditions. MitoTracker Red CMXRos was used as a fixable label for mitochondria. In the control condition, a small amount of punctuate staining pattern for NT was concentrated in the apical mitochondria of the OHCs, as shown in Figure 8B. The increased immunoreactivity for NT was detected both in the OHC cytoplasm and mitochondrial areas when animals were exposed to noise (Figure 8).

Discussion

The mitochondrial compartment of outer hair cells strongly labelled with DAF-2DA

Hair cells of the inner ear, as modified epithelial cells, are primarily detectors of sound. They carry highly

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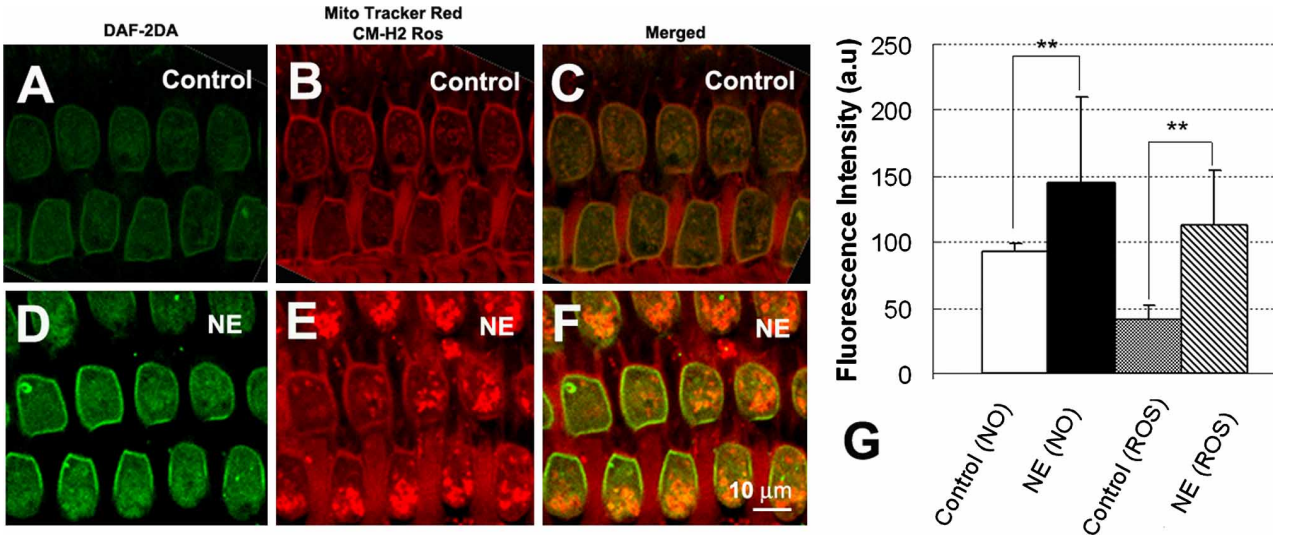

Figure 7. DAF-2DA and MitoTracker Red fluorescence change in OHCs of the organ of Corti from control and noise-exposed animals. (A) DAF-2DA fluorescence signals located at the mitochondria of the apical pole of OHCs from a control animal; (B) MitoTracker Red CM-H_2XRos fluorescence signals in the mitochondria of the OHCs of organ of Corti from the same control tissue; (C) A merged image from (A) and (B); (D) The increased DAF-2DA fluorescence signals at the mitochondria of the apical pole of OHCs from noise-stimulated tissue; (E) Elevated MitoTracker Red CM-H_2XRos fluorescence signals in the mitochondria of the OHCs from the same noise-exposed tissue; (F) A merged image from (D) and (E); (G) A graph of the changes of fluorescence and the significant difference in ROS production in the mitochondria of OHCs between control and noise exposed tissues. Data are means $\pm$SD ($n_{control}=66$ OHCs pooled from three specimens of three control animals; $n_{NE}=56$ OHCs pooled from three specimens of three noise-exposed animals, $p<0.05$).

specialized microvilli (the stereocilia) on their apical surface in the form of a bundle. Stereocilia bundle movement in response to sound vibrations gates an ionic current that depolarizes and activates the hair cell. Hair cells are morphologically polarized and a ring of tight junctions defines two major domains: an apical domain and a baso-lateral domain. As reported by Harada et al. [49], variously shaped mitochondria are predominantly located in the apical and basal poles of sensory hair cells. Mitochondrial function is fundamental to hair cell processes and involved in the normal and pathological physiology of sound transduction. In particular, the apical domain of the OHC has the energy load for calcium extrusion following entry via the mechano-transduction channel (by activity of PMCA in stereocilia) and for high membrane turnover and synthesis of apical domain proteins [37,38].

NO has been implicated in the regulation of mitochondrial respiration and cell apoptosis [50]. However, in the auditory system, the effects of NO on mitochondrial respiration and protein nitration in relation to the mitochondria of OHCs have not been studied. Using confocal fluorescence microscopy and two fluorescent dyes—DAF-2DA, which detects NO (by binding the chemical intermediate N_2O_3 formed from the reaction of NO and O_2) and the potential-dependent probe $TMRM^+$, a mitochondrial marker, we found that a high level of DAF-2DA fluorescence in OHCs was co-localized with mitochondria. This finding is consistent with the hypothetical production of NO by mtNOS (the existence of which is controversial [25]) as well as the NO produced by cytosol NOS that might diffuse to the mitochondria [26,51]. Since the amount of DAF-2DA signal is dependent on the binding of chemical intermediate N_2O_3 formed from the reaction of NO and O_2, in mitochondria under stress there could be a reduced amount of O_2 or conversion of NO to peroxinitrite by the reaction with superoxide. Both of these events would reduce DAF-2DA signal. Thus, one could interpret the signal we obtained as a minimum. Figure 6 shows that the OHC cytosol also has a significantly increased level of DAF-2DA fluorescence. This suggests that cytosolic NOSs are strongly stimulated by loud sound—perhaps from a calcium-dependent mechanism, since sound has been shown to increase OHC calcium [52,53]. Although the percentage increase of DAF-2DA is about the same for cytosolic and mitochondrial compartments (each about a 65% increase), the absolute level of photonic signal is higher for mitochondria. This supports the production of NO by mtNOS. This is evidence for a higher level of reactive nitrogen species production in the mitochondrial compartment and the subsequent protein nitration that could locally affect the mitochondria.

OHCs have three major isoforms of NOS (eNOS, nNOS and iNOS) that have been respectively identified in the hair bundles, the cuticular plates and the cytoplasm of the hair cells [27,28,54–58]. Mitochondrial NOS (mtNOS) has received considerable research support (see, for example, the review by Carreras et al. [24]) but remains controversial. Lacza et al. [25] believe that any unique mitochondrial isoform cannot be differentiated from cellular isoform contamination of the mitochondrial preparations.

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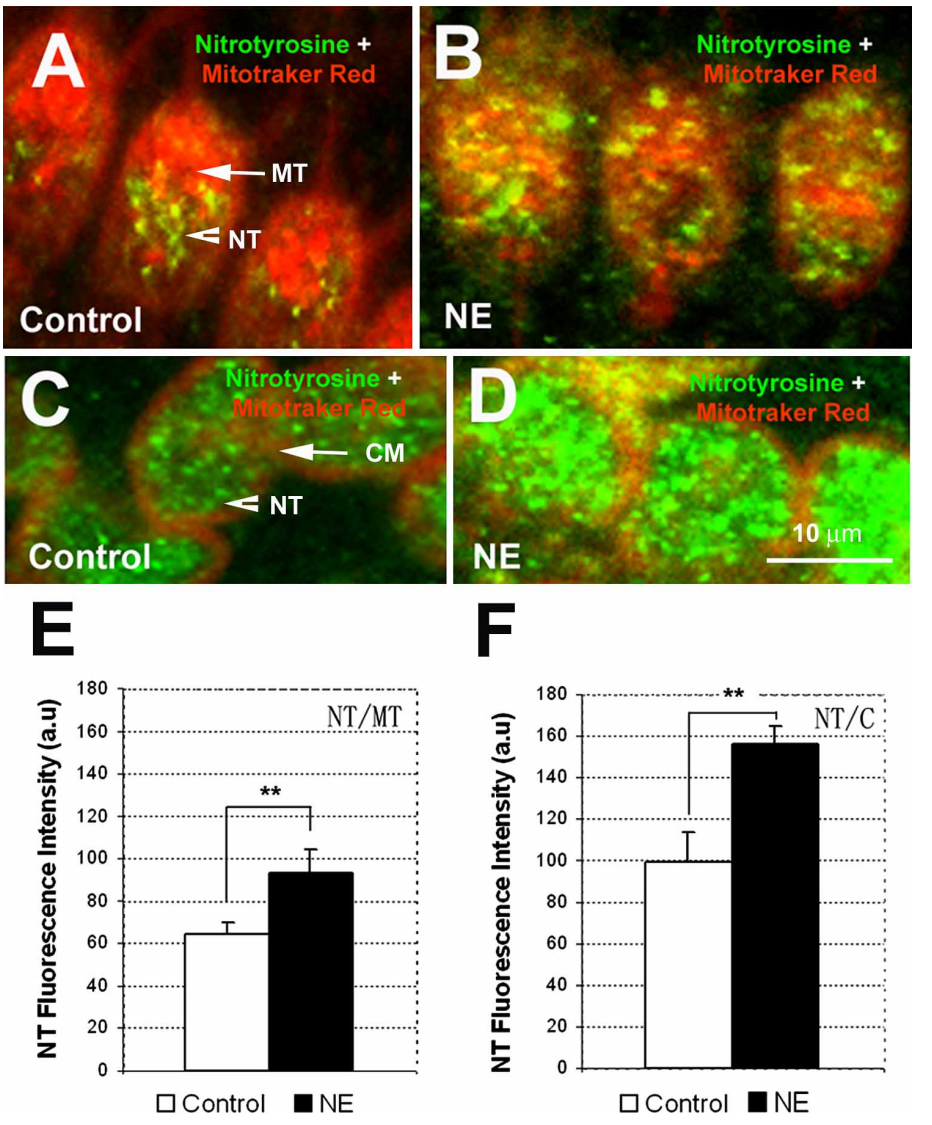

Figure 8. Representative single optical section confocal images of the fluorescent immunoreactivity of nitrotyrosine (NT) under control and noise exposure conditions. (A) A relatively small punctate staining pattern with anti-tyrosine antibody in the cytosole (arrow head) and mitochondria areas at the apical domain of OHCs (arrow). In contrast, an increased punctate staining pattern was observed in the mitochondrial area of OHCs after noise exposure (B). (C) The punctate staining pattern with anti-nitrotyrosine antibody in the cytosol (arrow head) under the control condition. However, anti-nitrotyrosine fluorescence increased substantially in the cytosol after noise stimulation. (E and F) A significantly increased mean level of nitrotyrosine label in both the mitochondria and cytosol of OHCs. Data are means ± SD ($n_{control}$ = 166 OHCs pooled from six specimens of six control animals; n_{NE} = 148 OHCs pooled from five specimens of five noise-exposed animals, $p < 0.01$; MT: mitochondria; CM: cell membrane).

Moreover, the level of NO and its regulation of OHC mitochondria have not been investigated. Since NO is a small, hydrophobic species with a diffusion coefficient similar to that of oxygen, its diffusion distance is ~140 μm [21]. Thus, NO within the mitochondria of the hair cell could originate from any of the types of NOS in the cell. Although the full biological function of NO in the OHC mitochondrion is not known, the DAF-2DA signal for NO within the mitochondrion of OHC suggests that NO may regulate energy metabolism, a role similar to its function in non-auditory cells such as in the brain and macrophages [44].

Mitochondrial membrane potential of the OHC was suppressed by exogenously applied NO above 300 nm concentration

The mitochondria of different tissues and cells have different sensitivities to NO and mitochondrial respiration is much more sensitive when tissues or cells undergo ischemia and conditions of lower oxygen concentrations [59]. Early studies from Brown and Borutaite [44,59] showed that the mitochondria from isolated nerve terminals could be reversibly inhibited at 250 nm of NO production; 300 nm NO could reversibly inhibit cytochrome

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oxidase activity in heart mitochondria [60]; 1 μm NO and 1 mm NO, respectively, irreversibly affected mitochondrial function in brain and macrophages [44]. In an earlier study, we found that 273 nm NO in the cochlear perilymph and noise cause NO production in the pericyte, up to 618 nm, damaging many but not all hair cells [32]. In this *in vitro* study, we found that ~350 nm NO (generated from 1 μm NOC-7) significantly collapsed the mitochondrial membrane potential of OHCs (as shown in Figure 4), which suggests that *in vitro* OHCs may have a similar sensitivity to NO regulation of respiration and of damage as have brain cells and macrophages, though *in vivo* OHC mitochondria may have a different vulnerability.

In pathological sound-stimulated conditions, perilymph NO can reach the threshold level, where mitochondrial membrane potential could be depolarized [32]. Measuring perilymph levels of NO would likely lead to an under-estimation of the NO level that the mitochondria in OHC would be exposed to. Thus, mitochondrial dysfunction by rapid and irreversible inhibition of many mitochondrial components, including most elements of complex I [24,61,62] is a possible result from loud sound stimulation. The long-term depolarization of mitochondria would open the mitochondria permeability transition pore to release cytochrome *c* and other apoptosis factors [63], For a review see Le Bras et al. [64].

Mitochondrial ROS and nitrotyrosine increased after noise exposure

Mitochondria have been recognized as critical sources and targets of nitrating species [65]. An increased NO concentration and its related free radicals in the mitochondria could overwhelm mitochondrial natural antioxidant defenses [66,67] and cause local protein nitration, such as of MnSOD, aconitase, cytochrome *c*, voltage-dependent anion channel and ATPase, as well as succinyl-CoA oxoacid-CoA transferase [67,68].

In this study, we examined changes in the amount of free radicals in general and in particular nitrogen free-radical-related protein nitration in the OHC following noise exposure. We found significantly increased reactive free radicals (ROS) at the mitochondria by the increased level of MitoTracker Red CM-H_2XRos fluorescence. Also, the increase of DAF-2DA signal could be interpreted as evidence of free radical potentiation of NO binding, should there be no specific mtNOS [69,70]. Nevertheless, the high level of NO combined with mitochondrial-produced superoxide can result in excess mitochondrial oxidants in OHCs. This is consistent with our current and early findings and others showing that not only are NO-related free radicals found [9], but also that showed hydroxyl radical (4-hydroxy-2-nonel) damage to lipid membranes by lipid peroxidation, which significantly increases in the cochlea after noise trauma [8,10,71].

With co-labelling techniques (MitoTracker Red 568 combined with an antibody for nitrotyrosine), we found an increased label for nitrotyrosine in the mitochondria (as shown in Figure 7). This provides new evidence for the role of NO in pathology within the auditory sensory cells through direct mitochondrial damage following noise trauma. Significantly, cytochrome *c* contains four highly conserved tyrosine residues; two tyrosines are easily accessible for peroxides [72]. When cytochrome *c* is released from mitochondria, it is highly soluble in aqueous environments and could be nitrated and form part of the cytosol mitochondrial signal in apoptotic cells. To account for a basal level of nitrotyrosine, it is known that peroxynitrite can nitrate the tyrosine residue of other proteins in different cellular locations from a basal level of NO generation [73]. Thus, the significantly increased immunoreactivity of nitrotyrosine in the cytosol after noise stimulation could be due to (1) increased NO production in the cytosol combined with mitochondrial produced superoxide; (2) mitochondrial release and nitration of cytochrome *c*; or (3), as already mentioned, diffusion of peroxynitrite from mitochondria to the cytosol. Also mitochondria have strong enzymatic antioxidant defense systems, such as superoxide dismutase and a high concentration of glutathione [66,67,73]. In either case, this is evidence for a higher level of reactive nitrogen species production in the mitochondrial compartment causing subsequent protein nitration (indicated by NT immunolabel) that occurs locally to the mitochondria.

This study provides evidence for the mitochondrial production of NO and NO related free radicals that could be responsible for a decreased mitochondrial potential of OHC, following damaging sound exposures. Moreover the mitochondrial level of NO is related to increased protein nitration in both mitochondria and for cytosolic proteins and this may be evidence of early damage to mitochondria that ultimately will lead to release of proapoptotic cytochrome *c*.

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References

[1] Hyde GE, Rubel EW. Mitochondrial role in hair cell survival after injury. Otolaryngol Head Neck Surg 1995;113:530–540.

[2] Fischel-Ghodsian N, Kopke RD, Ge X. Mitochondrial dysfunction in hearing loss. Mitochondrion 2004;4:675–694.

[3] Vicente-Torres MA. A BAD link to mitochondrial cell death in the cochlea of mice with noise-induced hearing loss. J Neurosci Res 2006;83:1564–1572.

[4] Jiang H, Talaska AE, Schacht J, Sha SH. Oxidative imbalance in the aging inner ear. Neurobiol Aging 2006; Epub 2006 Aug 2018.

[5] Monsalve M, Borniquel S, Valle I, Lamas S. Mitochondrial dysfunction in human pathologies. Front Biosci 2007;1:12.

[6] Yamane H, Nakai Y, Takayama M, Konishi K, Iguchi H, Nakagawa T, Shibata S, Kato A, Sunami K, Kawakatsu C. The emergence of free radicals after acoustic trauma and strial blood flow. Acta Otolaryngol Suppl 1995;519:87–92.

[7] Husbands JM, Steinberg SA, Kurian R, Saunders JC. Tip-link integrity on chick tall hair cell stereocilia following intense sound exposure. Hear Res 1999;135:135–145.

[8] Ohinata Y, Miller JM, Schacht J. Protection from noise-induced lipid peroxidation and hair cell loss in the cochlea. Brain Res 2003;966:265–273.

[9] Shi XR, Nuttall AL. Upregulated iNOS and oxidative damage to cochlear stria vascularis due to noise stress. Brain Res 2003;967:1–10.

[10] Yamashita D, Jiang HY, Schacht J, Miller JM. Delayed production of free radicals following noise exposure. Brain Res 2004;1019:201–209.

[11] Henderson D, McFadden SL, Liu CC, Hight N, Zheng XY. The role of antioxidants in protection from impulse noise. Ann NY Acad Sci 1999;28:368–380.

[12] Van de Water TR, Lallemend F, Eshraghi AA, Ahsan S, He J, Guzman J, Polak M, Malgrange B, Lefebvre PP, Staecker H, Balkany TJ. Caspases, the enemy within, and their role in oxidative stress-induced apoptosis of inner ear sensory cells. Otol Neurotol 2004;25:627–632.

[13] Kopke RD, Jackson RL, Coleman JK, Liu J, Bielefeld EC, Balough BJ. NAC for noise: from the bench top to the clinic. Hear Res 2007;226:57–65. Epub 2006 Dec 20.

[14] Staecker H, Liu W, Malgrange B, Lefebvre PP, Van de Water TR. Vector-mediated delivery of bcl-2 prevents degeneration of auditory hair cells and neurons after injury. ORL J Otorhinolaryngol Relat Spec 2007;69:43–50.

[15] Niu XZ, Shao R, Canlon B. Suppression of apoptosis occurs in the cochlea by sound conditioning. Neuroreport 2003;14:1025–1029.

[16] Nicotera TM, Hu BH, Henderson AJ. The caspase pathway in noise-induced apoptosis of the chinchilla cochlea. J Assoc Res Otolaryngol 2003;4:466–477.

[17] Han W, Shi X, Nuttall AL. AIF and EndoG translocation in noise exposure induced hair cell death. Hear Res 2005;211:85–95.

[18] Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol 2006;147(Suppl 1):S193–S201.

[19] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424.

[20] Ghafourifar P, Bringold U, Klein SD, Richter C. Mitochondrial nitric oxide synthase, oxidative stress and apoptosis. Biol Sign Recep 2001;10:57–65.

[21] Valdez LB, Zabornyj T, Boveris A. Mitochondrial metabolic states and membrane potential modulate mtNOS activity. Biochim Biophys Acta 2006;1757:166–172. Epub 2006.

[22] Cooper CE, Giulivi C. Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology. Am J Physiol Cell Physiol 2007;292: C1993–C2003. Epub 2007 Feb 28.

[23] Haynes V, Elfering S, Traaseth N, Giulivi C. Mitochondrial nitric-oxide synthase: enzyme expression, characterization, and regulation. J Bioenerg Biomemb 2004;36:341–346.

[24] Carreras MC, Franco MC, Peralta JG, Poderoso JJ. Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. Molec Aspects Med 2004;25:125–139.

[25] Lacza Z, Pankotai E, Csordas A, Gero D, Kiss L, Horvath EM, Kollai M, Busija DW, Szabo C. Mitochondrial NO and reactive nitrogen species production: does mtNOS exist? Nitric Oxide 2005;14:162–168. Epub 2005 Jul 26.

[26] Davidson SM, Yello DM. The role of nitric oxide in mitochondria. Focus on 'Modulation of mitochondrial Ca2+ by nitric oxide in cultured bovine vascular endothelial cells'. Am J Physiol Cell Physiol 2005;289:C775–C777.

[27] Shi X, Dai C, Nuttall AL. Altered expression of inducible nitric oxide synthase (iNOS) in the cochlea. Hearing Res 2003;177:43–52.

[28] Heinrich U-R, Selivanova O, Feltens R, Brieger J, Mann W. Endothelial nitric oxide synthase upregulation in the guinea pig organ of Corti after acute noise trauma. Brain Res 2005;1047:85–96.

[29] Murashita H, Tabuchi K, Hoshino T, Tsuji S, Hara A. The effects of tempol, 3-aminobenzamide and nitric oxide synthase inhibitors on acoustic injury of the mouse cochlea. Hear Res 2006;214:1–6.

[30] Heinrich UR, Maurer J, Gosepath K, Mann W. Immune electron microscopic detection of nitric oxide synthases I and III and synaptophysin in the organ of Corti in the guinea pig. Eur J Cell Biol 1997;74:99.

[31] Mitchell C, Kempton B, Creedon T, Trune DR. Rapid acquisition of auditory brainstem responses with multiple frequency and intensity tone bursts. Hear Res 1996;99:38–46.

[32] Shi XR, Ren T, Nuttall AL. The electrochemical and fluroescence detetection of nitric oxide in the cochlea and its increase following loud sound. Hear Res 2002;164:49–58.

[33] Floryk D, Houstk J. Tetramethyl rhodamine methyl ester (TMRM) is suitable for cytofluorometric measurements of mitochondrial membrane potential in cells treated with digitonin. Biosci Rep 1999;19:27–34.

[34] Julian D, April KL, Patel S, Stein JR, Wohlgemuth SE. Mitochondrial depolarization following hydrogen sulfide exposure in erythrocytes from a sulfide-tolerant marine invetebrate. J Exp Biol 2005;208:4109–4122.

[35] Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. Trends Neurosci 2000;23:166–174.

[36] Scaduto RC, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 1999;76:469–477.

[37] Griesinger CB, Richards CD, Ashmore JF. Apical endocytosis in outer hair cells of the mammalian cochlea. Eur J Neurosci 2004;20:41–50.

[38] Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, Corwin JT, Corey DP. Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 2003;23:4054–4065.

[39] LeMasurier M, Gillespie PG. Hair cell mechanotransduction and cochlear amplification. Neuron 2005;48:403–415.

[40] Shanker G, Syversen T, Aschner JL, Aschner M. Modulatory effect of glutathione status and antioxidants on methylmercury-induced free radical formation in primary cultures of cerebral astrocytes. Brain Res Mol Brain Res 2005;137:11–22. Epub 2005 Mar 2017.

[41] Nishio K, Qiao S, Yamashita H. Characterization of the differential expression of uncoupling protein 2 and ROS production in differentiated mouse macrophage-cells (Mm1) and the progenitor cells (M1). J Mol Histol 2005;36:35–44.

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[42] Yamakura F, Ikeda K. Modification of tryptophan and tryptophan residues in proteins by reactive nitrogen species. Nitric Oxide 2005;14:152–161.
[43] Poot M, Zhang Y, Kramer J, Wells K, Jones L, Hanzel D, Lugade A, Singer V, Haugland R. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J Histochem Cytochem 1996;44:1363–1372.
[44] Brown GC, Borutaite V. Nitric oxide, cytochrome c and mitochondria. Biochem Soc Symp 1999;66:17–25.
[45] Brown GC, Borutaite V. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. Free Radic Biol Med 2002;33:1440–1450.
[46] Ghafourifar P, Cadenas E. Mitochondrial nitric oxide synthase. Trends Pharmacol Sci 2005;26:190–195.
[47] Larstad M, Soderling AS, Caidahl K, Olin AC. Selective quantification of free 3-nitrotyrosine in exhaled breath condensate in asthma using gas chromatography/tandem mass spectrometry. Nitric Oxide 2005;13:134–144.
[48] Rodrigo J, Fernandez AP, Serrano J, Peinado MA, Martinez A. The role of free radicals in cerebral hypoxia and ischemia. Free Radic Biol Med 2005;39:26–50.
[49] Harada Y, Sakai T, Tagashira N, Suzuki M. Intracellular structure of the outer hair cell of the organ of Corti. Scan Electron Microsc 1986;(Pt 2):531–535
[50] Brown GC. NO says yes to mitochondria. Science 2003;299:838–839.
[51] Giulivi C, Kato K, Cooper CE. Nitric oxide regulation of mitochondrial oxygen consumption I: celllular physiology. Am J Physiol Cell Physiol 2006;291:C1225–C1231. Epub 2006 Aug.
[52] McDermott BMJ, Lopez-Schier H. Inner ear: Ca(2+)n you feel the noise? Curr Biol 2004;14:R231–R232.
[53] Shen H, Zhang B, Shin J, Lei D, Du Y, Gao X, Wang Q, Ohlemiller KK, Piccirillo J, Bao J. Prophylactic and therapeutic functions of T-type calcium blockers against noise-induced hearing loss. Hear Res Hear Res 2007;226:52–60.
[54] Fessenden JD, Coling DE, Schacht J. Detection and characterization of nitric-oxide synthase in the mammalian cochlea. Brain Res 1994;668:9–15.
[55] Hess A, Bloch W, Arnhold S, Andressen C, Stennert E, Addicks K, Michel O. Nitric oxide synthase in the vestibulocochlear system of mice. Brain Res 1998;813:97–102.
[56] Heinrich UR, Lioudyno M, Maurer J, Mann W, Guth PS, Forstermann U. Localization of the two constitutively expressed nitric oxide synthase isoforms (nNOS and eNOS) in the same cell types in the saccule maculae of the frog Rana pipiens by immunoelectron microscopy: evidence for a back-up system? J Elec Microsc 2003;52:197–206.
[57] Heinrich UR, Maurer J, Mann W. Evidence for a possible NOS back-up system in the organ of Corti of the guinea pig. Eur Arch Oto-Rhino-Laryngol 2004;261:121–128.
[58] Gosepath K, Gath I, Maurer J, Pollock JS, Amedee R, Forstermann U, Mann W. Characterization of nitric oxide synthase isoforms expressed in different structures of the guinea pig cochlea. Brain Res 1997;747:26–33.
[59] Bourutaite V, Brown GC. Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem J 1996;315:295–299.
[60] Poderoso JJ, Peralta JG, Lisdero CL, Carreras MC, Radisic M, Schopfer F, Cadenas E, Boveris A. Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. Am J Physiol Cell Physiol 1998;274:C112–C119.
[61] Sarti P, Arese M, Bacchi A, Barone MC, Forte E, Mastronicola D, Brunori M, Giuffre A. Nitric oxide and mitochondrial complex IV. Iubmb Life 2003;55:605–611.
[62] Haynes V, Elfering SL, Squires RJ, Traaseth N, Sohen J, Ettl A, Giulivi C. Mitochondrial nitric-oxide synthase: Role in pathophysiology. Iubmb Life 2003;55:599–603.
[63] Armstrong JS. The role of the mitochondrial permeability transition in cell death. Mitochondrion 2006;6:225–234.
[64] Le Bras M, Rouy I, Brenner C. The modulation of inter-organelle cross-talk to control apoptosis. Med Chem 2006;2:1–12.
[65] Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med 2002;33:1451–1464.
[66] Drake J, Sultana R, Aksenova M, Calabrese V, Butterfield DA. Elevation of mitochondrial glutathione by -glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. J Neurosci Res 2003;74:917–927.
[67] Bartesaghi S, Ferrer-Sueta G, Peluffo G, Valez V, Zhang H, Kalyanaraman B, Radi R. Protein tyrosine nitration in hydrophilic and hydrophobic environments. Amino Acids 2007;32:501–15. Epub 2006 Nov 2.
[68] Aulak KS, Koeck T, Crabb JW, Stuehr DJ. Dynamics of protein nitration in cells and mitochondria. Am J Physiol Heart Circ Physiol 2004;286:H30–H38.
[69] Roychowdhury A, Luthe G, Keihoff G, Wolf G, Horn TF. Oxidative stress in glial cultures: detection by DAF-2 fluorescence used as a tool to measure peroxynitrite rather than nitric oxide. Glia 2002;38:103–114.
[70] Jourd'heuil D. Increased nitric oxide-dependent nitrosylation of 4,5-diaminofluorescein by oxidants: implications for the measurement of intracellular nitric oxide. Free Radic Biol Med 2002;33:676–684.
[71] Ohlemiller KK, McFadden SL, Ding DL, Flood DG, Reaume AG, Hoffman EK, Scott RW, Wright JS, Putcha GV, Salvi RJ. Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. Audiol Neurootol 1999;4:237–246.
[72] Batthyany C, Zouza JM, Duran R, Cassina A, Cervenansky C, Radi R. Time course and site(s) of cytochrome c tyrosine nitration by peroxynitrite. Biochemistry 2005;44:8038–8046.
[73] Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci USA 2004;101:4003–4008.

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